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Long-acting β2 adrenergic agonists (LABAs) provoke Th17-biased asthma

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by

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TABLE OF CONTENTS

LIST OF FIGURES

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ABSTRACT OF THE THESIS

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Master of Science in Biology

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Professor Eyal Raz, Chair
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Asthma is a heterogeneous disease with distinct features of airway hyper-responsiveness (AHR) and mucus secretion. In general, long-acting β2 adrenergic agonists (LABAs) are commonly recommended for asthma patients to reduce these symptoms. When LABAs act on airway smooth muscle (ASM), they bind to ASM's β2 adrenergic receptors (β2ARs) to increase cAMP, which induces bronchodilation effect to ameliorate the AHR. In our previous study, we observed that the G protein-coupled receptors (GPCRs) in dendritic cells (DCs) triggers the Th17

differentiation of naïve T lymphocytes via cAMP signaling. Such a pro-Th17 activity is a serious response because it induces neutrophilic asthma with more severe inflammation. Thus, LABAs, which directly target β2AR of both ASM and lung DCs, need re-evaluation for their risk of causing severe inflammation. Therefore, we examined whether LABAs trigger β2AR of lung DCs and activate Th17 differentiation in asthma. Our results demonstrated that LABA treatment induced Th17 response in both murine DCs and human DC-like cell lines *in vitro.* Also, we observed that LABA inhalation triggered neutrophilic asthma *in vivo.* After we had confirmed the side-effect of LABAs, we further examined the combination of LABAs with Inhaled corticosteroids (ICS). Our results from LABA-ICS combination administration showed that the LABA-ICS combination inhibited both Th2 and Th17 response in asthma, supporting the anti-inflammatory effect of ICS with LABAs. Conclusively, we showed that LABAs have a side-effect of inducing neutrophilic asthma with its Th17 response, which suggests that the continuing use of LABAs may worsen the asthmatic symptoms of LABAs users.

Chapter 1: Introduction

1.1 Asthma and long-acting β2 adrenergic agonists (LABAs)

Asthma is a chronic inflammatory disorder characterized by narrowing the airways and accumulating mucus (Brannan & Lougheed, 2012). It is a global health issue since the number of asthma patients exceeds 300 million, and 250,000 people die annually (Bousquet et al., 2010). The direct cause of asthma is still unknown, and various environmental and genetic factors are being examined. One of the main issues that make asthma dangerous is that it induces airway hyper-responsiveness (AHR), which derives excessive tightness of airways with an influx of immune cells (Brannan & Lougheed, 2012). The distinct feature of AHR is its inappropriate response to allergens. Various innate and adaptive immune cells, including mast cells, basophils, dendritic cells, and helper T lymphocytes, participate in the AHR by triggering pathophysiological changes in the epithelial membrane with unnecessary immune reactions (Borish, 2016). When airways get sensitized by allergens, immunoglobulin E (IgE) is produced and binds to mast cells and basophils (Madore & Laprise, 2010). These activations of mast cells and basophils lead to degranulation of proinflammatory mediators and cytokines that increase hyper-responsiveness and inflammation (He, Zhang, Zeng, Chen, & Yang, 2013). Helper $CD4^+$ T lymphocytes take a crucial role in asthma by recruiting other innate immune cells to the inflammation site and increasing the severity of inflammation (Irvin et al., 2014). The influx of mast cells and basophils to the AHR inflammation site is called eosinophilic inflammation, and T helper type 2 (Th2) cells, a classic asthmatic T lymphocyte, derives it (Irvin et al., 2014). Th2 attracts mast cells and basophils into airway smooth muscles (ASM) by releasing its distinct cytokines, such as IL-4 and IL-5 (Kay, 2006). Such an influx of immune cells intensifies asthma and causes abrupt airway constriction, which may

threaten patients' lives (Gaurav & Agrawal, 2013). Another form of helper T lymphocytes that takes an essential role in asthma is T helper type 17 (Th17) lymphocytes (Irvin et al., 2014). Th17-induced inflammation in the airway increases the number of neutrophils and macrophages, which directly affects the severity of asthma and has a risk of stimulating more mucus secretion and even permanent lung damages (Ponce-Gallegos, Ramirez-Venegas, & Falfan-Valencia, 2017). Neutrophilic Th17 response in asthma increases neutrophilic immune cells' saturation and eventually reduces asthma therapy response (Irvin et al., 2014).

A common therapy for asthma is administering bronchodilators, which help relaxing ASM and alleviating obstructed airway (Johnson, 2001). One of the prevailing asthma treatments is β2-adrenergic receptors (β2AR) agonists, which directly bind to the β2AR of ASM and induces bronchodilation via cAMP/PKA signaling (Johnson, 2001). There are two types of β2 adrenergic agonists; Short-acting β2 agonists (SABAs) and Long-acting β2 agonists (LABAs) (Barnes, 2010). SABAs are effective therapy for acute asthma with their rapid action. However, SABAs' half-life only lasts up to six hours. On the other hand, LABAs are commonly used for treating chronic asthma with their longer half-life, which lasts more than twelve hours (Barnes, 2011). However, LABAs became a controversial treatment because it was found to be an enhancer of deteriorated allergic asthma if administered for a long term without corticosteroids (Barnes, 2011). Thus, the United States Food and Drug Administration instructs that LABAs should always be co-treated with Inhaled corticosteroids (ICS). Yet, the mechanism of LABAs' side-effect in inducing inflammation, morbidity, and mortality has not been clearly defined.

1.2 G protein-coupled receptor (GPCR)

β2AR and its cAMP signaling are suspected of taking an essential role in LABAs' side-effects in asthmatic inflammation. β2AR belongs to a large family of transmembrane receptors, called G protein-coupled receptors (GPCR) (Kato et al., 2014). GPCR is acknowledged as the largest class of cell-surface transmembrane receptors with its participation in the number of cellular signaling cascades (Ghosh, Kumari, Jaiman, & Shukla, 2015). GPCR, among other activities, regulates the cAMP pathway through GPCR's heterotrimeric GTP binding proteins (αßγ) (Ghosh et al., 2015). Upon GPCR's activation via signaling molecules, GDP on the α subunit gets exchanged to GTP. By this exchange, G_α dissociates from the G protein complex and regulates enzymes, such as adenylyl cyclase (AC) (Ghosh et al., 2015). G proteins have four subfamilies based on their α subunit; G_s, $G_{i,}$ $G_{q,}$ and $G_{12/13}$ (Brauner-Osborne et al., 2006). Activation of α subunit of stimulating G-protein ($G_{\alpha s}$) stimulates AC, which catalyzes ATP to cAMP (Brauner-Osborne et al., 2006). Conversely, α subunit of inhibitory G-protein ($G_{\alpha i}$) inactivates AC and decreases the cAMP level. Secondly, there are two major ways of cAMP signaling: one is through the protein kinase A (PKA) pathway, and the other one is through the exchange protein of activated cAMP (EPAC) pathway (Lee et al., 2015). Thus, when ligands bind to β2AR, the cAMP level gets increased via $G_{\alpha s}$ and activates the cAMP-dependent PKA pathway for phosphorylation of various proteins, including cAMP response element-binding protein (CREB) (Rosenbaum, Rasmussen, & Kobilka, 2009). With these features, β2AR became a primary drug target with its ability to receive extracellular signals (Ghosh et al., 2015). With β2AR/cAMP pathway regulation, the β2AR agonists can provide bronchodilation of ASM as an asthma treatment. However, based on our recent research, we detected that β2AR agonists could promote severe inflammation with their cAMP pathway regulation in dendritic cells.

1.3 Activation of the cAMP signal pathway in dendritic cells (DCs)

In our previous study, we examined the elevation of cAMP in dendritic cells (DCs) (Lee et al., 2020). DCs are innate immune cells that are essential in connecting the innate immune system and adaptive immune systems (Condon, Sawyer, Fenton, & Riches, 2011). As antigen-presenting cells (APCs), DCs react to immunogens by presenting their antigens to T lymphocytes (Condon et al., 2011). Before T lymphocytes receive DCs' signals, T lymphocytes are in naïve form. Naïve helper T lymphocytes become effector helper T lymphocytes only when they are polarized by DCs' MHC-II-antigen complex (ten Broeke, Wubbolts, & Stoorvogel, 2013). Because of their role as antigen-presenting cells (APCs), DCs can also initiate allergic asthma by taking up allergens, processing them, and polarizing naïve T lymphocytes (Gaurav & Agrawal, 2013).

Classes of DCs are mainly divided into conventional DCs (cDCs), plasmacytoid DCs (pDCs), and monocyte-derived DCs (MoDCs) (Condon et al., 2011). cDCs usually reside in tissues and can be distinguished by their expression of CD11c integrin. This particular group can be sub-divided into cDC1 and cDC2 (Condon et al., 2011). cDC1 polarizes naïve helper T lymphocytes into T helper type 1 (Th1) lymphocytes and presents viral antigens to cytotoxic $CD8^+$ T lymphocytes (Plantinga et al., 2013). On the other hand, cDC2 promotes Th2 or Th17 differentiations of helper T lymphocytes, and these polarized Th2 and Th17 release cytokine signals to other immune cells to react against exogenous antigens (Vroman et al., 2015). MoDCs are another subset of DC families that are derived from monocytes. MoDCs mainly produce proinflammatory chemokines, and they can also induce Th2 response (Guilliams et al., 2014).

Based on cDC2's ability to promote Th2 or Th17 differentiation of helper T lymphocytes, we had postulated that cAMP upregulation through GPCR would change the Th2 response of cDC2 to Th17 response. Through our study of cDC2, we identified that cAMP regulation via DCs' GPCR could switch Th2-biased DCs to Th17-biased DCs (Lee et al., 2020). More specifically, we found that the upregulation of the cAMP/PKA pathway in cDC2 decreased *IRF4* expression, a transcription factor that is known for promoting Th2 differentiation of helper T lymphocytes (Huber & Lohoff, 2014). When *IRF4* expression of cDC2 was decreased, helper T lymphocytes were switched from Th2 to Th17 response (Lee et al., 2020). This experiment demonstrated that the induction of the cAMP/PKA pathway in DCs affected T lymphocytes to switch from Th2 response to Th17 response (Lee et al., 2020). Since it was shown that GPCR activation could upregulate the cAMP/PKA pathway of DCs, we suspected that β2AR agonist treatment for asthma might target unintended immune cells and make deteriorated asthmatic inflammation via cAMP/PKA signaling upregulation.

1.4 Evaluation of LABA and Inhaled corticosteroid (ICS) combination

For asthma patients, a combination of LABA and Inhaled corticosteroid (ICS) is strongly mandated for LABA administration (Barnes, 2010). Particularly, ICS is used to repress inflammation in asthma and mucus secretion (Solidoro, Patrucco, & Bagnasco, 2019). Not only it increases the anti-inflammatory effect, but ICS also restores β2AR's responsiveness to the β2 agonists by upregulating the expression of β2AR (Barnes, 2010). However, β2AR agonists' potential to develop side effects in asthma suggests that ICS also needs to be evaluated for its impact on DCs (Ramamoorthy & Cidlowski, 2016). Since ICS is known for inducing β2AR's responsiveness, there is a risk of developing pro-Th17 inflammation with the LABA-ICS

combination treatment as well. Although LABA-ICS combination treatment is strongly recommended in any asthma treatment to ensure such an anti-inflammatory along with LABAs' bronchodilation effect, the mechanism of LABA-ICS on reprogramming of DCs and thus switching Th2 to Th17 differentiation is still not clearly defined.

1.5 The effect of LABAs on asthma severity

Our previous study of DCs' role in reprogramming T lymphocytes endotypes suggests that the current bronchodilator treatments, such as SABAs and LABAs, need re-evaluation for their safety. Since GPCR of DCs is crucial in regulating cAMP concentration, inappropriate use of β2AR agonists has high possibilities of inducing Th17 differentiation and of causing more severe neutrophilic asthma.

In chapter 2, we investigated whether LABA treatment induces a neutrophilic Th17 response in the airway. First, we verified the effect of Formoterol (LABA) treatment on cAMP signaling and Th17 response by administering Formoterol on each ASM and bone-marrow DCs (BMDCs) of wild-type mice (C57B1/6). In addition to that, we also confirmed LABAs' effect on human cells by treating Formoterol on human DC-like monocyte cell lines: THP-1 (monocyte), HL-60 (promyeloblast), and MUTZ-3 (myelomonocyte). Also, we demonstrated the impact of LABAs on lung resident DCs' reprogramming by stimulating the cAMP signaling via Formoterol administration *ex vivo.* Secondly, to clarify whether LABAs directly induce Th17 differentiation and neutrophilic asthma *in vivo*, we applied Formoterol through intranasal (i.n) delivery to house dust mite (HDM)-sensitized mice *in vivo*. Additionally, to assess the effect of the LABA-ICS combination on asthma severity, we administered Formoterol with budesonide (ICS) to the asthma

models as well. We also investigated the duration of the LABA-ICS combination by re-sensitizing the asthma models one month after the last LABA-ICS administration. Conclusively, we aimed to evaluate whether LABA treatment induces Th17 response and eventually switches eosinophilic inflammation to neutrophilic inflammation in allergic asthma. Ultimately, these findings will suggest a new perspective of using β2AR agonists in asthma and a chance to develop a better amendment for today's prevailing asthma treatments.

Phenotypes of polarized T lymphocytes were analyzed through cytokine profiling by enzyme-linked immunosorbent assay (ELISA). DCs and T lymphocytes from both *in vitro* and *in vivo* experiments were analyzed by qPCR to evaluate their genetic expression under each condition.

1.6 Previous studies from our laboratory on addressing this topic

So far, our laboratory had devoted to finding the role of cAMP in DCs and helper T lymphocytes' immunity. First, we discovered that Cholera toxin (CT) promoted a mucosal immune response in mice by inducing Th17 response (Datta et al., 2010). Additionally, we examined the important role of cAMP in helper T lymphocytes' differentiation by regulating the $G_{\alpha s}$ of helper T lymphocytes (Li et al., 2012). Based on these observations, we found that the cAMP concentrations in DCs determined the regulation of Th2 response (Lee et al., 2015). Also, we demonstrated that the induction of cAMP concentration in DCs switched helper T lymphocytes from Th2 to Th17 responses (Lee et al., 2020). Currently, we are evaluating the immunological impact of LABAs *in vitro* and *in vivo* in models of allergic asthma, and the materials of this thesis are part of the current research in preparation for submission in early 2021.

Chapter 2: Results

2.1 LABA treatment on BMDCs induces Th17 response of helper T lymphocytes via cAMP/PKA signaling pathway *in vitro.*

LABAs have long been used in asthma to induce bronchodilation for easier breathing. LABAs mainly regulate ASM's cAMP level by targeting β2ARs, which are extensively distributed throughout the lung and predominate over other βARs (Mutlu & Factor, 2008). Our previous study found that cAMP agonists influence DCs and increase Th17 response by upregulating the cAMP/PKA signaling pathway (Lee et al., 2020). So, we suspected that LABA treatment in the airway might trigger lung resident DCs' reprogramming and provoke Th17 response of helper T lymphocytes, which can cause neutrophilic inflammation.

To confirm that LABAs induce β2AR-dependent responses in both DC and ASM, we examined Formoterol (LABA) treatment targeting β2AR of BMDCs of WT mice (C57B1/6). First, we compared the frequency of β2ARs expression on BMDCs and on ASM. BMDCs and ASM were collected from WT mice. In fact, both BMDCs and ASM expressed a high level of *Adrb2* mRNA, a gene that encodes β2AR (Fig. 1.1a). Among βARs, *Adrb2* mRNA was the highest in BMDCs (Fig. 1.1a). Moreover, the *Adrb2* mRNA expression of BMDCs was 3-folds higher than that of ASM (Fig. 1.1a). Through Fluorescence-activated cell sorting (FACS) analysis, we also confirmed that the β2AR expression of BMDC was 2-folds higher than that of ASM (Fig. 1.1b). To verify that Formoterol treatment regulates cAMP signaling of BMDCs, BMDCs and ASM were treated with Formoterol and forskolin, an AC activator. The cAMP assay illustrated that Formoterol increased cAMP signaling levels in both BMDCs and ASM (Fig. 1.1c). Collectively,

these results suggest that BMDCs express a sufficient amount of β2ARs as much as ASM does, and Formoterol is capable of inducing cAMP signaling of both ASM and BMDCs.

After we had confirmed the sufficient β2AR expression of BMDCs, we examined whether LABA treatment activates BMDCs' Th17-biased reprogramming through β2AR/cAMP signaling. WT BMDCs were treated with Formoterol for 2, 6, and 16 hours duration, and their Th2-inducing transcription factors, *Irf4* and *Klf4*, were measured through qPCR analysis. *Crem* was also measured to ensure the elevation of cAMP. The Formoterol treatment derived the reduction of *Irf4/Klf4* and the induction of *Crem* (Fig. 1.2a). Such a result shows that the Formoterol treatment inhibits *Irf4/Klf4* through β2AR/cAMP signaling. We further tested Formoterol treatment on BMDCs to confirm that its β2AR/cAMP signaling induces PKA to diminish *Irf4*. Thus, we treated PKA inhibitor (Rp-cAMP), EPAC inhibitor (CE3F4), and CREB inhibitor (666-15) along with Formoterol delivery on BMDC. When PKA inhibitor was treated with Formoterol, BMDCs' *Irf4* expression recovered (Fig. 1.2b). On the other hand, the EPAC inhibitor did not revert the effect of Formoterol (Fig. 1.2b). CREB, which is a downstream effector of PKA, confirmed the effect of the β2AR/cAMP/PKA pathway by increasing *Irf4* expression (Fig. 1.2b). This result supports that LABAs reprogram BMDCs to become Th17-biased through β2AR/cAMP/PKA signaling pathway.

So, we further tested whether the reprogrammed BMDCs induce Th17 differentiation of helper T lymphocytes via coculture of WT BMDCs with splenic OT2 $CD4^+$ T lymphocytes. OT2 mice are transgenic models that are made to have specific T cell receptors (TCRs), which only recognize the OVA antigens presented by MHC class II of DCs (Leung et al., 2012). By loading OVA peptides to BMDCs prior to the coculture, we were able to get a higher response of helper T

lymphocytes' differentiations that are specific to the BMDCs. Then, we treated Formoterol on WT BMDCs along with other various β2AR agonists, including short-acting β2AR agonists, Salmeterol (Sal), and Epinephrine (Epi) on BMDCs. Additionally, we treated cAMP analog (CPT) and PRR-dependent pathogen molecule (Curdlan, as control) to compare the level of cAMP of BMDCs in each condition. Then, the BMDCs were pulsed with OVA peptide, washed, and cocultured with OT2 $CD4^+$ T lymphocytes for three days. These OT2 $CD4^+$ T lymphocytes were anti-CD3/anti-CD28 restimulated to harvest their cytokines. Cytokine profiles of the effector OT2 $CD4^+$ T lymphocytes revealed that all treatments had elevated the T lymphocytes' IL-17A, which is an indicator of Th17 differentiation (Fig. 1.3a). When Formoterol was treated, IL-4 cytokine profile, an indicator of Th2 differentiation, did not increase significantly in the cocultured T lymphocytes (Fig. 1.3b). IFNγ, an indicator of Th1 differentiation, also remained unchanged (Fig. 1.3b). Moreover, when BMDCs were treated with βAR antagonists, Propranolol (Pro) and ICI-118,551(ICI), IL-17A cytokine profile of the cocultured T lymphocytes did not increase (Fig. 1.3c). We further identified the expression changes of the T lymphocytes' lineage commitment factor genes through qPCR analysis. Each lineage of effector T lymphocytes (Th1, Th2, Th17, and regulatory T lymphocytes) express *T-bet, GATA3, RORγt,* and *Foxp3*, respectively. The test showed a significant increase in *RORγt* level (Th17 phenotype inducer gene) when Formoterol was treated (Fig. 1.3d). Other T-lymphocyte lineage commitment factor genes (*GATA3*, *T-bet*, and *Foxp3*) demonstrated no statistical difference for both untreated and Formoterol-treated conditions (Fig. 1.3d). These results suggest that BMDCs' reprogramming via Formoterol induced the Th17 response of T lymphocytes.

Collectively, both ASM and BMDCs have sufficient β2ARs to activate the cAMP/PKA signaling pathway. Also, Formoterol treatment on BMDC reduced *IRF4* and *KLF4* TF via the β2AR/cAMP/PKA pathway. This Formoterol effect induced Th17 response of helper T lymphocytes. Therefore, LABA treatment on BMDCs can stimulate Th17 immunity by reprogramming DC to Th17-biased.

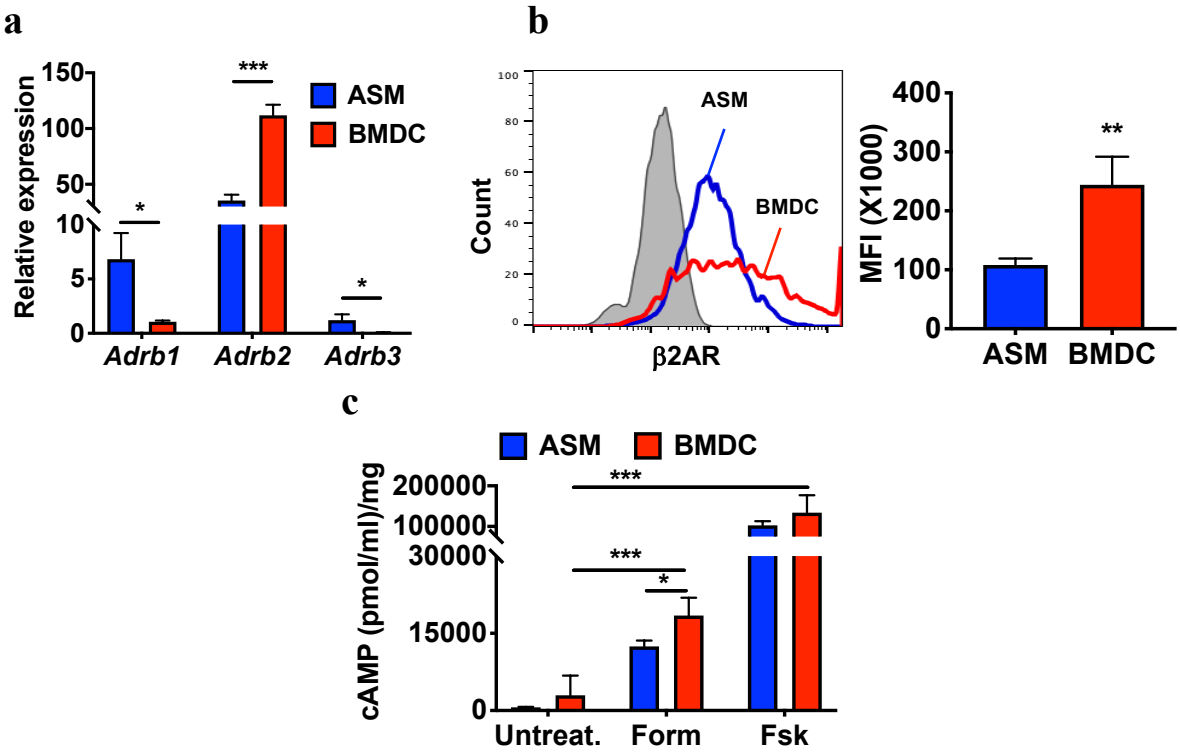


Figure 1.1. Formoterol induced the cAMP signaling of both ASM and BMDCs. (a) qPCR analysis of *Adrb1*, *Adrb2*, and *Adrb3* relative expressions in ASM and BMDC. **(b)** FACS analysis of β2AR expression in ASM and BMDC. **(c)** cAMP assay result of ASM and BMDC with Form (Formoterol, 25 μM) treatment and Fsk(Forskolin, 10 μM) as a positive control. Two-way ANOVA with Sidak's multiple comparison test, n=3 in each group, * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

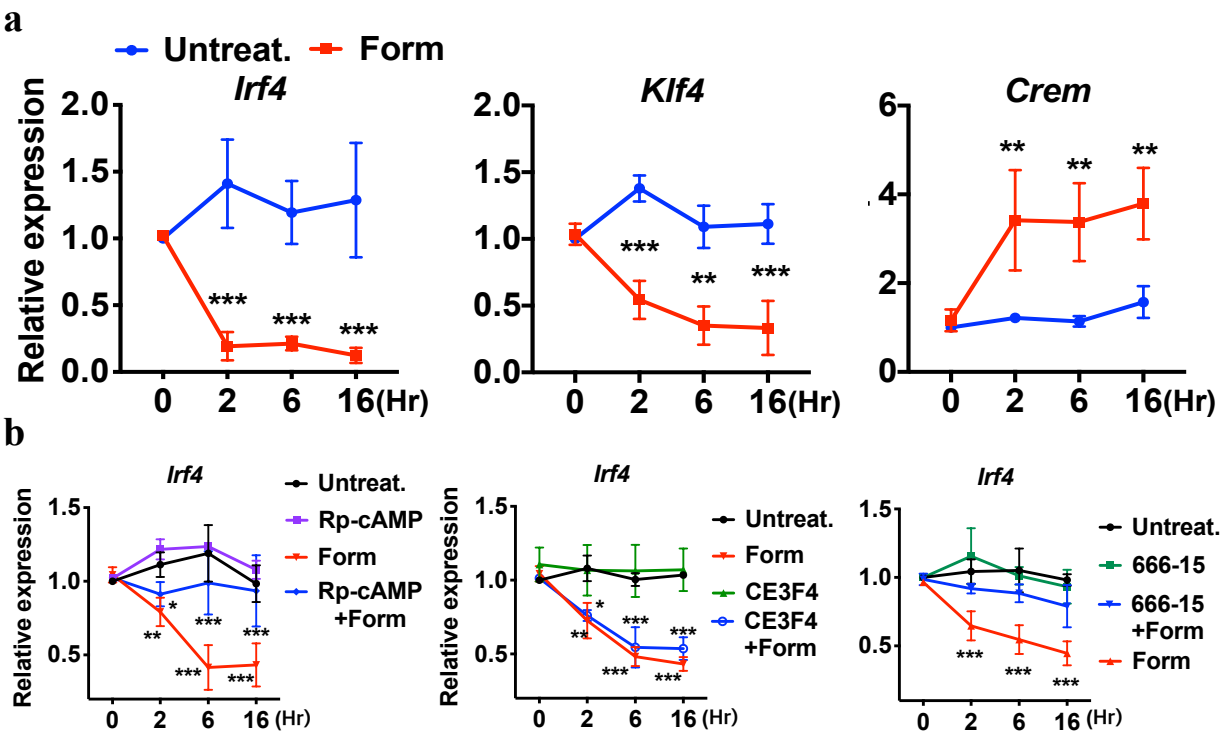


Figure 1.2. Formoterol reduced Th2-biasing TFs via β2AR/cAMP/PKA signaling pathway. **(a)** qPCR analysis of the relative expressions of BMDC TFs (*Irf4, Klf4,* and *Crem)* from WT BMDCs that had been treated with Formoterol (25μM). **(b)** qPCR analysis of the relative expressions of *Irf4* from WT BMDCs that had been treated with each PKA inhibitor (Rp-cAMP), EPAC inhibitor (CE3F4), and CREB inhibitor (666-15) for 6 hours. After the 6 hours, Formoterol (25μM) was treated. Two-way ANOVA with Sidak's multiple comparison test, n=3 in each group, * p<0.05, ** p<0.01, ***p<0.001.

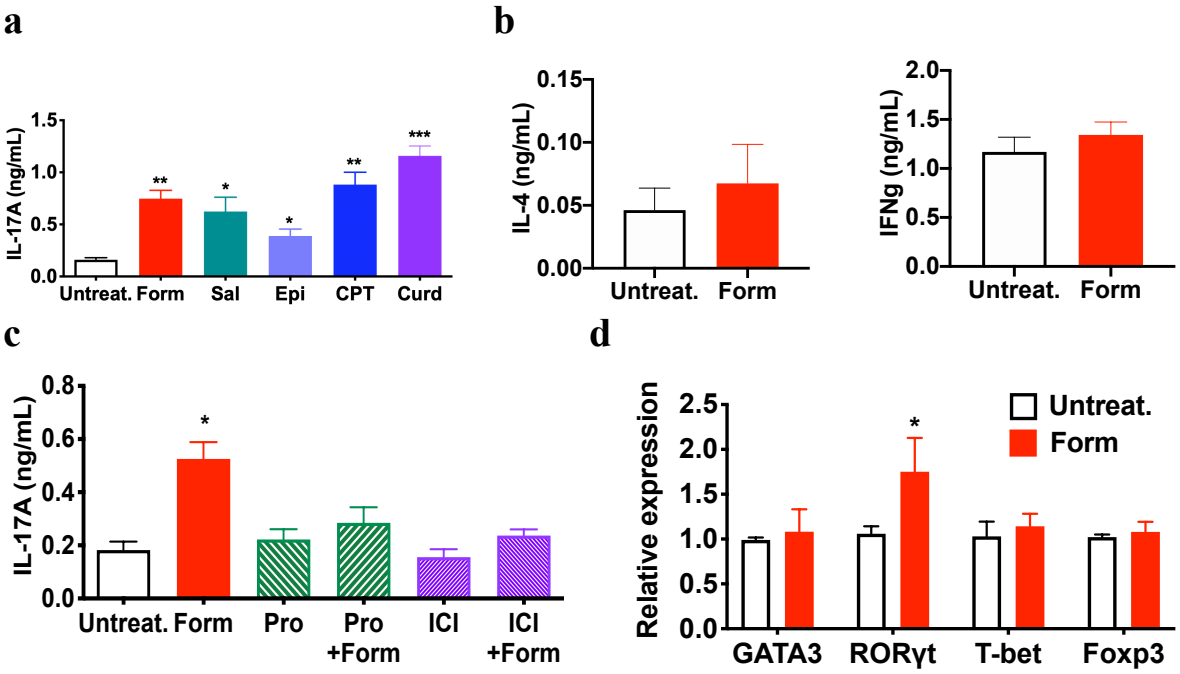


Figure 1.3. BMDCs' reprogramming via Formoterol induced Th17 response of T lymphocytes. (a) Cytokine (IL-17A) levels of the anti-CD3/anti-CD28 restimulated the OT2 $CD4^+$ T lymphocytes, which had been co-cultured with WT BMDCs. The WT BMDCs were pulsed with OVA peptides and and treated with β2AR agonists, including Formoterol (Form), Salmeterol (Sal), and Epinephrine (Epi). cAMP analog (CPT) and PRR-dependent pathogen molecule (Curdlan) were also treated to WT DCs. **(b)** Cytokines (IL-4 and IFNγ) levels of the anti-CD3/anti-CD28 restimulated OT2 $CD4^+$ T lymphocytes, which had been loaded with OVA peptides and cocultured with untreated and Formoterol-treated WT BMDCs. **(c)** Cytokine (IL-17A) level of the anti-CD3/anti-CD28 restimulated OT2 $CD4^+$ T lymphocytes, which had been loaded with OVA peptides and cocultured with untreated and Formoterol-treated WT BMDCs. The WT BMDCs were also treated with β-AR antagonist (Propranolol, Pro), and β2AR selective antagonist (ICI-118,551). **(d)** qPCR analysis of the relative expressions of four different lineage commitment genes of the helper T lymphocytes for both untreated and Formoterol-treated conditions. The lineage commitment genes (*GATA3, RORγt, T-bet,* and *Foxp3*) represent differentiations of Th2, Th17, Th1, and Regulatory T-lymphocyte (Treg), respectively. Two-way ANOVA with Sidak's multiple comparison test, n=3 in each group, * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

2.2 LABAs reprogram human DC-like cell lines via cAMP/PKA signaling pathway *in vitro*.

So far, LABA treatment on murine BMDCs induced Th17 response of helper T lymphocytes via β2AR/cAMP/PKA signaling. From this founding, we postulated that LABAs would induce Th17-biasing reaction in human DCs as well. Hence, we investigated the effect of LABAs on human DCs by treating Formoterol on human DC-like monocyte cell lines: THP-1, HL-60, and MUTZ-3.

First, we examined whether the human DC-like cell lines express sufficient β2AR. We assessed the *ADRB2* mRNA expression of each cell line through qPCR and confirmed that all THP-1, HL-60, and MUTZ-3 express similar *ADRB2* mRNA (Fig. 2.1a). Through FACS analysis of each human DC-like cell line, we confirmed the expression of β2AR in THP-1, HL-60, and MUTZ-3 (Fig. 2.1b). The cAMP assay of the three cell lines had shown that Formoterol increased cAMP signaling levels in all THP-1, HL-60, and MUTZ-3 (Fig. 2.1c). After we had shown that the sufficient β2AR of THP-1, HL-60, and MUTZ-3 allows Formoterol to increase cAMP signaling, we further investigated whether Formoterol induces human DCs' Th17-biased reprogramming via β2AR/cAMP signaling. Thus, THP-1, HL-60, and MUTZ-3 were treated with Formoterol for 2, 6, and 16 hours duration. The cytokine profiles of the three cell lines demonstrated that their *IRF4* and *KLF4* gradually decreased with Formoterol treatment (Fig. 2.1d). *IRF8* level, which is a Th1-biasing indicator, was consistent, but *CREM* increased in all three cell lines (Fig. 2.1d). To assure that Formoterol activates the β2AR/cAMP/PKA pathway to diminish *IRF4*, we treated Formoterol along with PKA inhibitor (Rp-cAMP), EPAC inhibitor (CE3F4), and CREB inhibitor (666-15) on HL-60, one of the cell lines that expressed the highest Ardb2 mRNA. PKA inhibitor and CREB inhibitor repressed Formoterol's *IRF4* reducing effect (Fig. 2.1e).

However, the EPAC inhibitor did not recover the *IRF4* from the Formoterol's effect (Fig. 2.1e). This result suggests that the reduction of *IRF4* with Formoterol treatment was β2AR/cAMP/PKA-dependent.

In summary, all THP-1, HL-60, and MUTZ-3 cell lines expressed sufficient β2ARs that can trigger the cAMP/PKA signaling. Additionally, Formoterol treatment diminished IRF4 and KLF4 TFs via the β2AR/cAMP/PKA pathway in human DC-like cell lines. These results support that LABAs might trigger the Th17 response of helper T lymphocytes if LABAs act on human lung DCs.

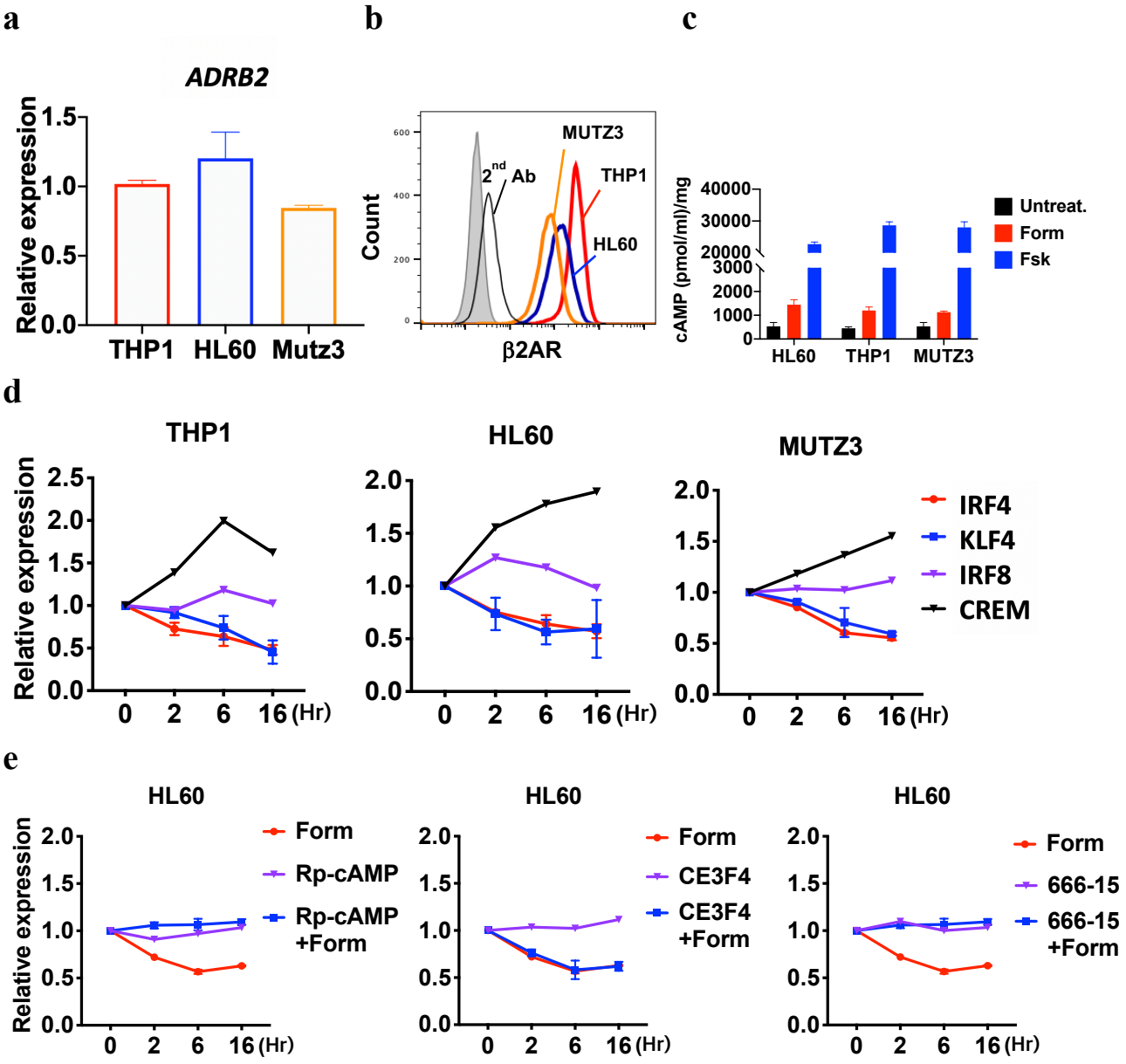


Figure 2.1. Formoterol induced cAMP signaling and reduces Th2-biasing TFs in human DC-like cell lines. (a) qPCR analysis of *ADRB2* mRNA relative expressions in three different human-DC like monocyte cell lines: THP-1, HL-60, and MUTZ-3. **(b)** FACS analysis of β2AR expression in THP-1, HL-60, and MUTZ-3. **(c)** cAMP assay result of THP-1, HL-60, and MUTZ-3 with Formoterol (25 μM) treatment and Fsk (10 μM) as a positive control. **(d)** qPCR analysis of the relative expressions of TFs (*IRF4*, *KLF4, IRF8*, and *CREM*) of human-DC like cell lines (THP-1, HL-60, and MUTZ-3) with Formoterol treatment. **(e)** qPCR analysis of the relative expression of *IRF4* in HL-60 with Formoterol and/or cAMP pathway inhibitors: PKA inhibitor (Rp-cAMP), EPAC inhibitor (CE3F4), and CREB inhibitor(666-15).

2.3 LABA treatment on lung cDC2 and MoDC populations triggers Th17 response of T lymphocytes *ex vivo.*

Lung resident DCs are composed of pDCs, cDCs, and MoDCs. Mainly, cDC2 and MoDCs induce Th2-derived eosinophilic inflammation in asthma (Vroman et al., 2015). We had previously demonstrated that LABA treatment promoted BMDCs' reprogramming in murine BMDCs and human DC-like cell lines. However, it is still not proved that LABAs can also reprogram lung resident DCs. Thus, we postulated that LABA treatment on the lung resident DCs would switch Th2 response to Th17 response via β2AR/cAMP/PKA signaling.

First, we performed HDM intranasal sensitization on WT mice for two weeks to provoke lung inflammation (Fig. 3.1a). Then, HDM-sensitized lung DCs were isolated and sorted into three DC subsets: $CD103^+$ cDCs ($CD11c^+CD103^+$), $CD11b^+$ cDCs ($CD11c^+CD103^-CD11b^+CD64^-$), and MoDCs ($CD11c^+CD103^-CD11b^+CD64^+$) through FACS (Fig. 3.1a). $CD103^+$ cDCs indicate cDC1, while $CD11b^+$ cDCs represent cDC2. The three subsets were treated with Formoterol for 0 to 16 hours. The level of Th2 priming TFs expressions, *Irf4* and *Klf4*, were reduced $CD11b^+$ cDCs and MoDCs but remained unchanged for $CD103^+$ cDCs (Fig. 3.1b). *Irf8* expression, a Th1 priming TF, remained unchanged for all subsets (Fig. 3.1b). The elevation of *Crem* in all subsets indicated the elevation of cAMP signaling with Formoterol treatment (Fig. 3.1b). To confirm the effect of Formoterol, each DC subset was treated with Formoterol, pulsed with OVA peptide, and cocultured with OT2 $CD4^+$ T lymphocytes. The coculture of three DC subsets with OT2 T lymphocytes differentiated the naïve T lymphocytes to the effector T lymphocytes. The cytokine from differentiated T cells was harvested after restimulation and analyzed through ELISA. The three subsets' cytokine profiles demonstrated a relatively strong IL-4 reduction and strong IL-17A

elevation in $CD11b^+$ cDCs and MoDCs (Fig. 3.1c). The lineages of differentiated effector T lymphocytes were identified by analyzing the lineage commitment gene expressions through qPCR. The test showed a relatively high increase in *RORγt* level (Th17 phenotype inducer gene) in the restimulated T cells from the Formoterol-treated $CD11b^+$ cDC and Formoterol-treated MoDC (Fig. 3.1d). Another T-lymphocyte lineage commitment factor gene, *GATA3*, demonstrated no statistical difference for both untreated and Formoterol-treated cases in all three subsets.

Collectively, LABAs increased cAMP signaling for both lung resident cDC2 and MoDCs, which diminished their IRF4 and KLF4 TFs. Eventually, the reprogramming of lung DCs polarized the Th17 response of helper T lymphocytes. Therefore, these results suggest that LABA treatment on lung resident DCs shifted Th2-biased to Th17-biased in the asthmatic model.

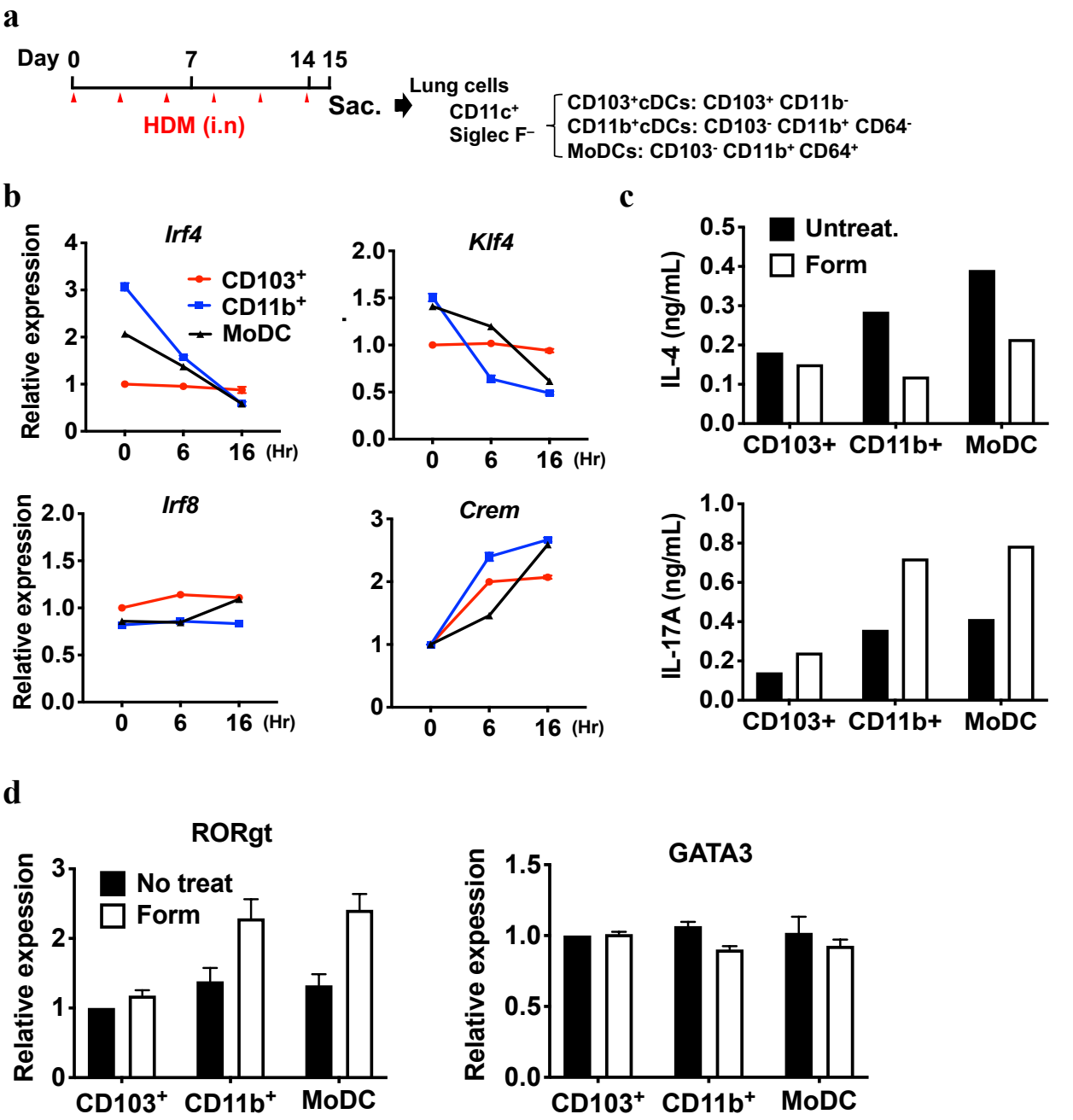


Figure 3.1. Formoterol treatment inhibited Th2 inducing TFs and increased Th17 cytokines in lung cDC2 and MoDC populations. (a) The protocol of HDM sensitization induced asthma model **(b)** qPCR analysis of the relative expression of TFs (*Irf4, Klf4, Irf8* and *Crem*) in Formoterol-treated lung resident cDCs (CD103$^+$, CD11b$^+$, and MoDC). **(c)** Cytokine (IL-4 and IL-17A) profiles of the anti-CD3/anti-CD28 restimulated OT2 CD4$^+$ T lymphocytes, which had been cocultured with lung-resident cDCs: CD103$^+$, CD11b$^+$ and MoDC. These lung-resident cDCs were pulsed with OVA peptides and treated with Formoterol. **(d)** qPCR analysis of the relative expressions of Th17 and Th2 lineage commitment genes of the cocultured OT2 helper T lymphocytes. The lineage commitment genes (*RORγt* and *GATA3*) represent differentiations of Th17 and Th2, respectively. Data are mean ± s.e.m; n =12, * p<0.05, ** p<0.01.

2.4 Intranasal LABA administration generates neutrophilic Th17 response in airway asthmatic model *in vivo*.

After we had verified that LABA treatment triggered Th17 response in lung resident DCs of WT mice, we hypothesized that intranasal LABA administration would affect Th17 differentiation of helper T lymphocytes in asthma patients' airways. Because of the Th17 response's tendency to neutrophilic asthma, we also postulated that the LABA administration might trigger shifting from eosinophilic response to neutrophilic response. To examine this hypothesis, the chronic asthma model was developed with HDM sensitization. Prior to Formoterol treatment, HDM was used to create allergic asthma reactions. Later, Formoterol was treated, and the models were observed whether its HDM-induced Th2 response switch to Th17 response or not.

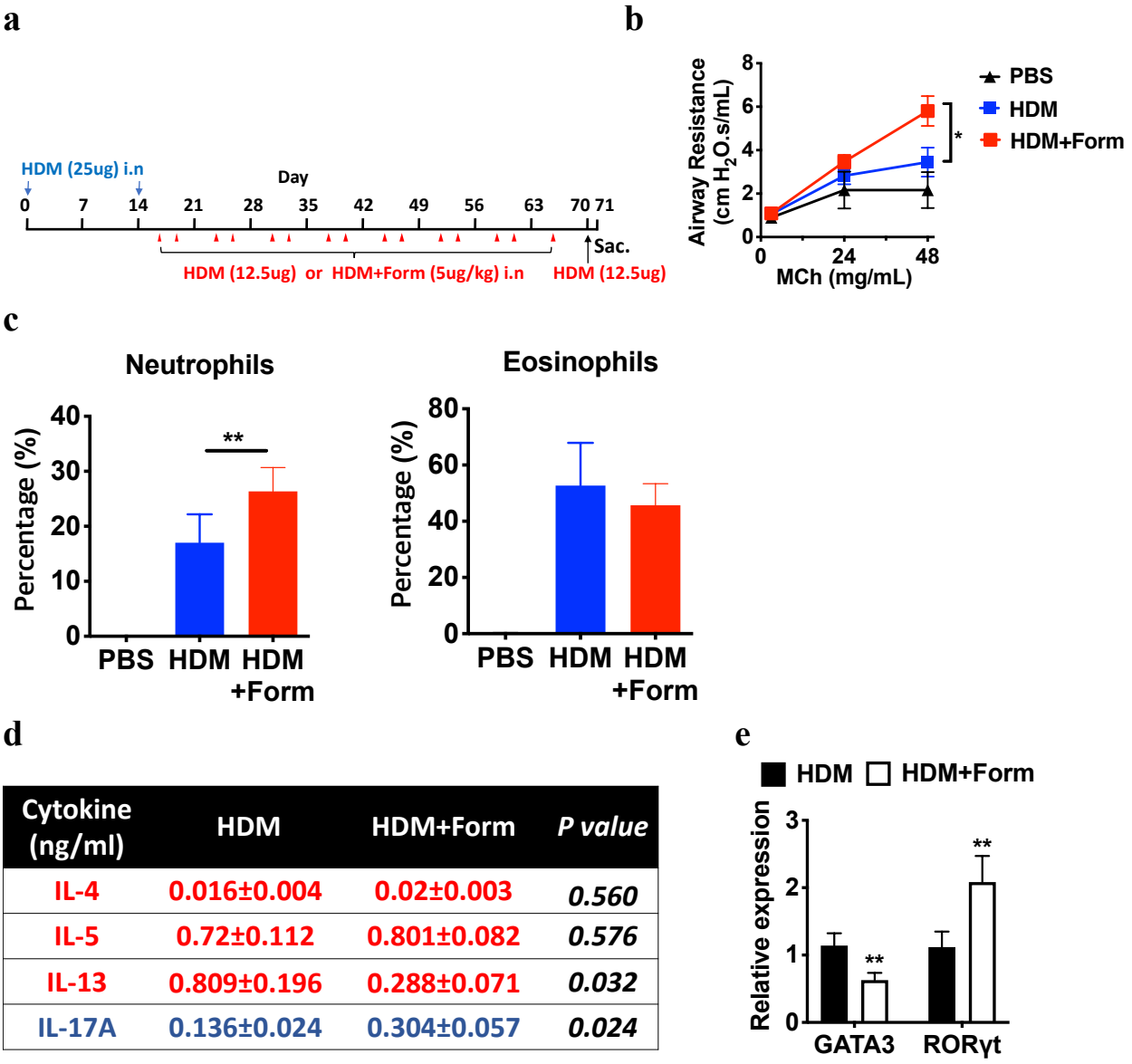
First, we developed an asthma model by sensitizing WT mice (C57BL/6) with two intranasal administration of HDM. These mice were re-exposed to HDM or HDM with Formoterol twice a week (Fig. 4.1a). After eight weeks of re-sensitization, we re-sensitized the mice with HDM to recall the immune response. Methacholine (MCh) challenge test was performed to evaluate the asthma models' breathing ability. The MCh challenge showed that the Formoterol treatment induced higher airway resistance than the control, demonstrating a significant increase of AHR in the model's airway (Fig. 4.1b). To observe the concentration change of neutrophils and eosinophils in lung airways, we performed bronchoalveolar lavage (BAL) in the airways with Hematoxylin and Eosin (H&E) staining. The result from BAL showed a higher percentage of neutrophils in the Formoterol treatment than the untreated control (Fig. 4.1c). The MCh challenge and BAL results were verified by the cytokine profile from the single-cell suspension of the mice models' lung tissue. IL-4, IL-5, and IL-13 levels, which are Th2 response markers, had shown a

decreasing trend in HDM with Formoterol condition. Meanwhile, IL-17A level significantly increased in the Formoterol treatment compared to the HDM-only condition (Fig. 4.1d). To confirm this Th17 response, we examined relative expressions of T cell lineage markers of the single-celled lung tissue. The T cell lineage marker analysis confirmed that the Th17 marker gene, *RORγt*, was significantly upregulated when Formoterol was treated, while the Th2 marker gene, *GATA3*, was significantly downregulated (Fig. 4.1e). These results support that intranasal Formoterol treatment provoked Th17-biased neutrophilic immunity in airways.

To visualize the switching to neutrophilic asthma phenotype, the asthma models' lung tissues were histologically analyzed. Hematoxylin & Eosin (H&E) staining revealed an increased number of inflammatory white blood cells (Fig. 4.2a). Also, Trichrome and Periodic Acid Schiff (PAS) stainings illustrated that the Formoterol treatment had increased collagen and mucus secretion (Fig. 4.2a). We supported the histological analysis result with qPCR analysis of *Muc5ac* mRNA of each HDM and the HDM + Formoterol models' lungs. *Muc5ac* mRNA expression is an indicator of heightened mucin production, and HDM + Formoterol model showed significantly higher expression of *Muc5ac* mRNA (Fig. 4.2b). The histological data and *Muc5ac* mRNA expression results suggest that intranasal Formoterol treatment intensified mucus secretion and airway remodeling with increased collagen.

The asthma models obtained pro-Th2 immunity when they were sensitized via HDM only. However, when they were re-sensitized by HDM + Formoterol, the phenotype of allergic asthma had been switched from eosinophilic inflammation to neutrophilic inflammation. Even worse, the models with Formoterol inhalation obtained more severe symptoms, such as mucus hyper-

secretion and airway remodeling. Collectively, Formoterol intranasal delivery induced neutrophilic asthma with increased mucus secretion and airway remodeling.



Cytokine (ng/ml)	HDM	HDM+Form	*P value*
IL-4	0.016±0.004	0.02±0.003	*0.560*
IL-5	0.72±0.112	0.801±0.082	*0.576*
IL-13	0.809±0.196	0.288±0.071	*0.032*
IL-17A	0.136±0.024	0.304±0.057	*0.024*

Figure 4.1. Intranasal Formoterol administration generated the elevation of neutrophils and induced Th17 cytokines in airway. (a) The protocol of HDM sensitization and challenge model. HDM (12.5 μg) or HDM + Form (Formoterol, 5μg) were treated through i.n. twice a week for 8 weeks on WT Mice (C57BL/6). **(b)** Airway resistance results of MCh in mice lungs with HDM and HDM + Form sensitizations. **(c)** Immune cell composition (%) of the BAL for each HDM and HDM + Form conditions. **(d)** Cytokine (IL-4, IL-5, IL-13 and IL-17A) profiles of lung cells in HDM and HDM + Form conditions. **(e)** qPCR analysis of the relative expressions of helper T lymphocytes lineage commitment genes for HDM and HDM + Form conditions. The lineage commitment genes (*GATA3* and *RORγt*) represent differentiations of Th2 and Th17 respectively. Data are mean ± s.e.m, n=6 mice per experiment in each group; * p<0.05, ** p<0.01.

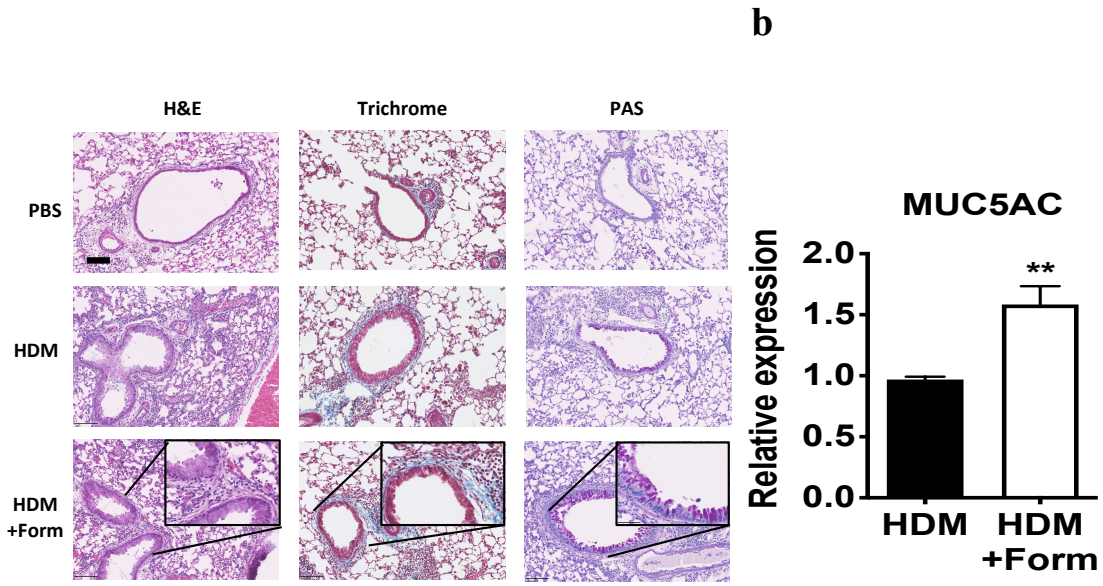


Figure 4.2. Intranasal Formoterol administration heightened mucus secretion and airway remodeling. (a) Lung tissues of HDM and HDM + Form administered mice were analyzed by histology: H&E, Trichrome, and PAS staining with magnification ×100, scale bar: 100 μm. **(b)** qPCR analysis of *Muc5ac* mRNA expressions from HDM or HDM + Form treated samples. Data are mean ± s.e.m, *n*=6 mice per experiment in each group; * $p<0.05$, ** $p<0.01$.

2.5 Combination of LABA and ICS inhalation on murine lungs reduced both Th2-derived and Th17-derived inflammation i*n vivo.*

So far, we had confirmed that LABAs construct Th17-mediated neutrophilic inflammation via the β2AR/cAMP/PKA pathway of DCs. According to the United States Food and Drug Administration, LABAs are mandated to be treated with ICS to reduce LABAs' side-effect. Although ICS is well-known for suppressing inflammation in asthma, the effect of the LABA-ICS combination on lung DCs and Th17 differentiation was not thoroughly examined (Barnes, 2010). Thus, we seek to verify whether ICS can prevent LABAs' Th17-mediated inflammation by reducing both the Th2 response and Th17 response in asthma. Again, the chronic asthma model was developed with HDM sensitization for Formoterol-Budesonide (LABA-ICS) combination treatment.

To understand whether the combination treatment of LABA and ICS reduces both Th2 and Th17 differentiation through DCs' cellular mechanisms, we sensitized WT mice (C57BL/6) by two intranasal HDM deliveries. Following the sensitization, HDM, HDM with Formoterol, or HDM with Formoterol-Budesonide was treated for eight weeks (Fig. 5.1a). AHR of each condition was measured by a MCh challenge test. The MCh challenge resulted in a significant increase in airway resistance in HDM + Formoterol condition (Fig. 5.1b). While HDM + Formoterol treatment worsened AHR from HDM sensitization, the HDM + Formoterol + Budesonide treatment significantly decreased the elevated asthmatic symptom (Fig. 5.1b). BAL had shown that the addition of Budesonide in Formoterol treatment had significantly decreased the number of neutrophils and eosinophils (Fig. 5.1c). These results support that the Formoterol-Budesonide combination treatment on murine lung reduced airway resistance and airway inflammation.

Moreover, we checked the cytokine profile of single-cell suspended lung tissues to observe whether the decrease in neutrophils and eosinophils is related to Th2 and Th17 differentiation. Both cytokines, IL-17a and IL-4, significantly decreased when Budesonide was treated with Formoterol (Fig. 5.2a). In the previous study, we confirmed that Formoterol treatment-induced *Muc5ac* mRNA expression led to the elevation of airway mucus hyper-secretion (Kraft, Cassell, Pak, & Martin, 2002). To show that Budesonide alleviates the Formoterol-induced inflammation, we assessed a histology test on lung tissues and analyzed *Muc5ac* mRNA expression of lung tissues through qPCR. While lung histology of Formoterol treatment showed intensified collagen deposition and mucosal metaplasia through airway remodeling, Budesonide's lung histology illustrated overall reduction of collagen and mucus. (Fig. 5.2b). qPCR analysis of the relative expression of *Muc5ac* mRNA confirmed the reduced mucus hyper-secretion in Formoterol-Budesonide combination (Fig. 5.2c). These results suggest the Budesonide reduced both Th2 and Th17 mediated inflammation and prevented AHR.

Budesonide is often treated for the long term to maximize its interaction with LABAs (Barnes, 2010). However, whether Budesonide treatment can maintain its anti-inflammation effect in the airway after stopping its treatment is not clearly defined. Thus, we measured the recovery of inflammation by suspending a long period usage of Budesonide. So, we designed another model that follows the same protocol of Formoterol-Budesonide combination treatment for 8 weeks but added a new condition where we re-exposed the mice to HDM a month later from the last Formoterol-Budesonide combination. After that, we checked whether Th17 or Th2 responses recover when the Formoterol-Budesonide combination is not treated for a month. We repeated the sensitization of WT mice by two intranasal HDM deliveries. Following the sensitization,

Budesonide was treated with Formoterol for eight weeks (Fig. 5.3a). After eight weeks, mice were untreated for one month. MCh challenge test showed that Formoterol's effect decreased, while the AHR in Budesonide treatment relatively increased (Fig. 5.3b). BAL demonstrated that one month of treatment prevention recovered the percentage of neutrophils and eosinophils in lung tissue compared to the percentage of neutrophils and eosinophils in the regular condition. (Fig. 5.3c). Although the cytokine profile showed IL-17a and IL-4 levels' recovery, the significant differences between ICS treatment and the control were maintained (Fig. 5.3d).

In summary, LABA-ICS combination delivery not only provided the airway constriction relief but also reduced both neutrophilic and eosinophilic inflammation. These results were verified by reduced Th2 and Th17 biasing cytokines and reduced mucus hyper-secretion. However, stopping the Formoterol-Budesonide treatment had proven its transient effect by worsening the alleviated bronchoconstriction and recalling the numbers of neutrophils and eosinophils. Therefore, these results support that the combination of LABA and ICS is effective in preventing LABAs' activation of Th17 response.

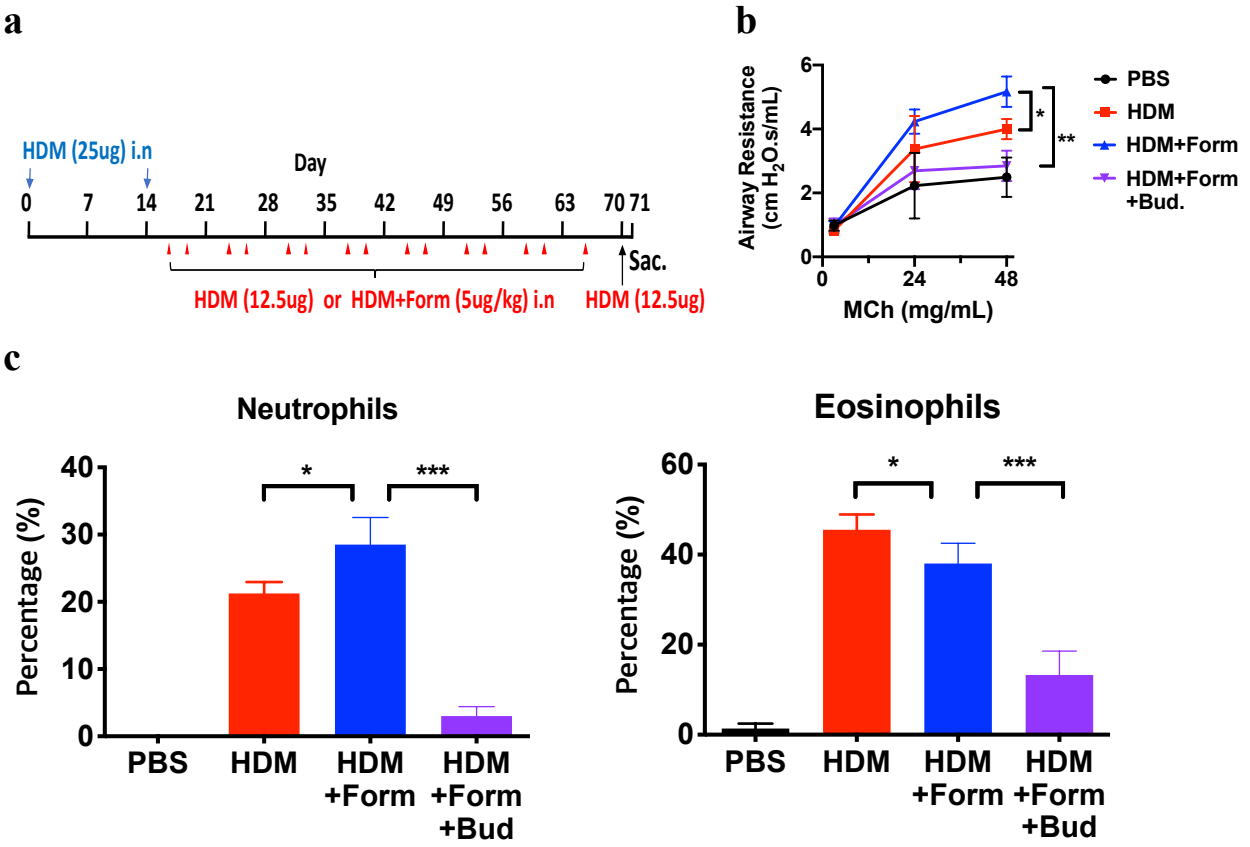


Figure 5.1. Combination of Formoterol and Budesonide administration on murine lungs reduced airway resistance and neutrophils/eosinophils concentrations. (a) The protocol of HDM sensitization and challenge model. HDM (12.5µg), HDM + Form (5µg), or HDM + Form + Bud (Budesonide, 85µg) were treated through i.n. twice a week for 8 weeks on WT Mice (C57BL/6). **(b)** Airway resistance results of MCh in mice lungs with HDM, HDM + Form, and HDM + Form + Bud sensitizations. **(c)** Immune cell composition (%) of the BAL fluid of each HDM, HDM + Form, and HDM + Form + Bud conditions. Data are mean ± s.e.m, *n*=6 mice per experiment in each group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

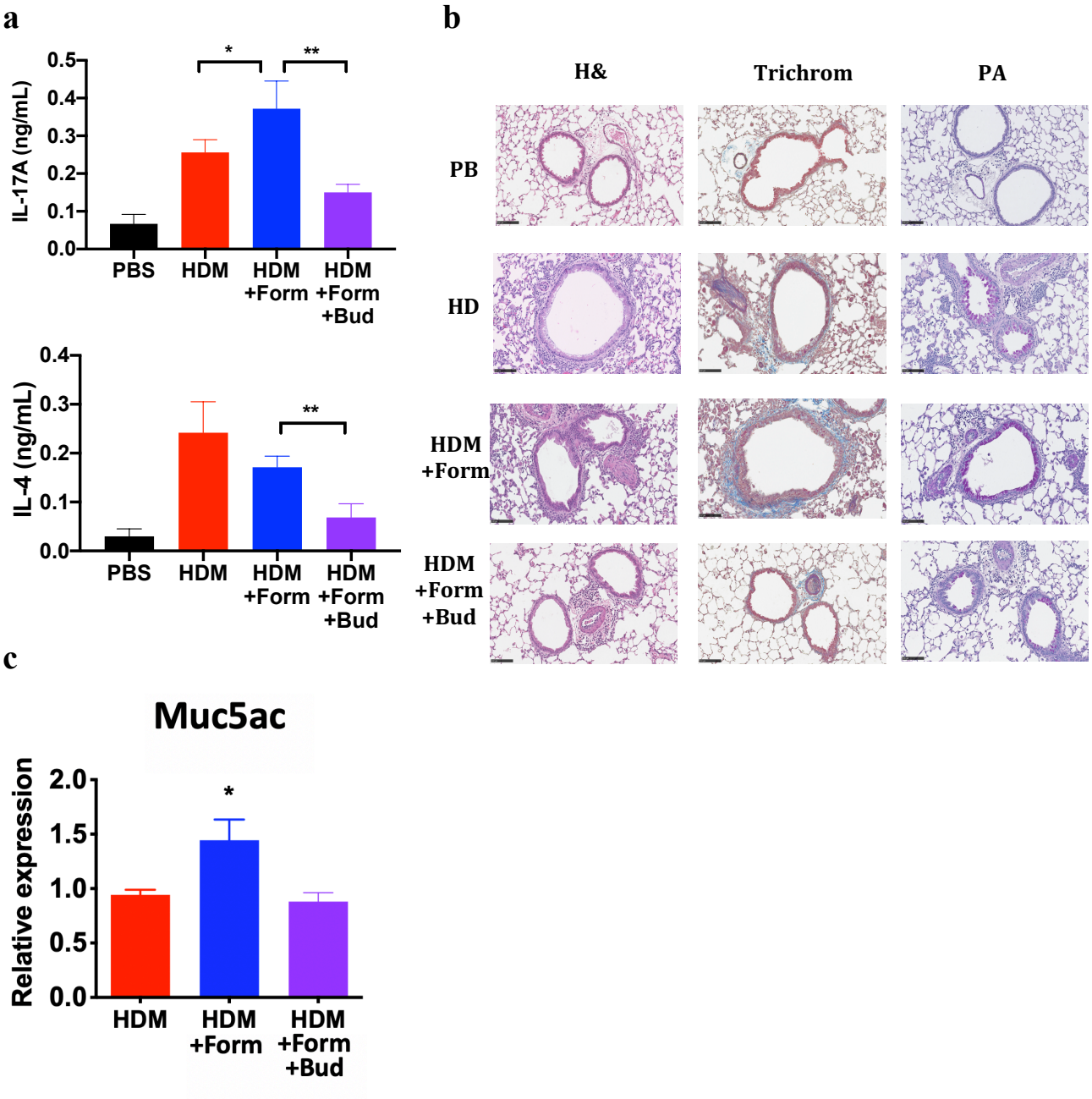


Figure 5.2. Combination of Formoterol and Budesonide administration on murine lungs reduced both Th2/Th17 response, airway remodeling, and mucus secretion. (a) Cytokine (IL-4 and IL-17A) profiles of single-cell suspended lung tissue of each conditions: HDM, HDM + Form, and HDM + Form + Bud. **(b)** Histological samples of each conditions of lung tissues are compared in H&E, blue-Trichrome and red-purple-PAS with magnification x100, scale bar:100 µm. **(c)** qPCR analysis of the *Muc5ac* mRNA expressions of HDM, HDM + Form, and HDM + Form + Bud samples. Data are mean ± s.e.m, n=6 mice per experiment in each group; * p<0.05, ** p<0.01.

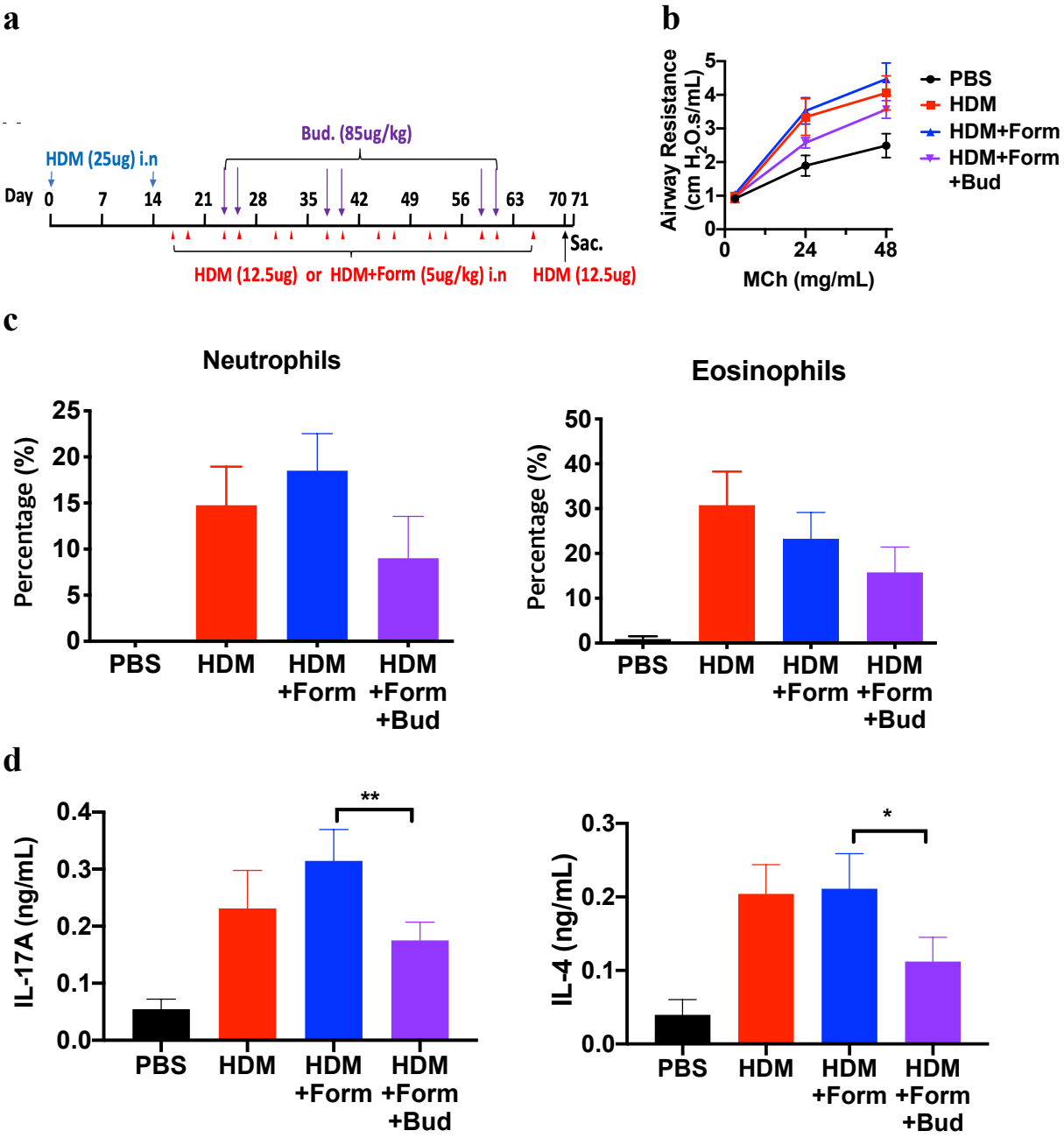


Figure 5.3. Stopping combination of Formoterol and Budesonide administration for one month recovered airway resistance, neutrophils/eosinophils concentrations, and Th2/Th17 cytokines. (a) The protocol of HDM sensitization and challenge model. HDM (12.5 µg), HDM + Form (5 µg), or HDM +Form + Bud (85 µg) were treated through i.n. twice a week for 8 weeks on WT Mice. The mice were resensitized after one month with HDM. **(b)** Airway resistance results of MCh in mice lungs with HDM, HDM + Form, and HDM + Form + Bud sensitizations. **(c)** Immune cell composition (%) of the BAL for each condition. **(d)** Cytokine (IL-4 and IL-17A) profiles of single-cell suspended lung tissue of each conditions: HDM, HDM + Form, and HDM + Form + Bud. Data are mean ± s.e.m, n=6 mice per experiment in each group; * p<0.05, ** p<0.01.

Chapter 3: Discussion and Future Studies

3.1 Discussion

One of the main asthma mechanisms of asthma is its classic allergic response with Th2 differentiation (Barnes, 2010). Th2-induced asthma causes an eosinophilic response, which eventually obstructs airways with AHR and mucus secretion (Knight et al., 2015). Among many drug options, LABAs are often used by asthma patients for better breathing of air with its bronchodilation effect (Knight et al., 2015). Nonetheless, LABAs' regulation of the cAMP level can also act on DCs that reside in patients' lungs and activate neutrophilic Th17 response that causes severe asthma.

Throughout the experiment, Formoterol consistently influenced lung DCs' cAMP/PKA pathway and induced pro-Th17 responses *in vitro* and *in vivo.* First, we showed that the regulation of the cAMP concentration in BMDCs affected helper T-lymphocyte differentiation via stimulating the β2AR/cAMP/PKA pathway with Formoterol. We further demonstrated that Formoterol reprogrammed Th2 inducing TF of human DC-like cell lines. Since the inhibitions of *IRF4* and *KLF4* indicated the Th17 response of helper T lymphocytes, the results show that the Formoterol treatment could bias Th17 response in human cells as well (Lee et al., 2020). Secondly, we discovered that Formoterol also elicited the cAMP/PKA pathway of lung resident DCs and reprogrammed the lung resident DCs to promote Th17 response of helper T lymphocytes. Thus, we also performed intranasal Formoterol delivery to the HDM-sensitized models and verified that the Formoterol administration remodeled eosinophilic asthma to neutrophilic asthma *in vivo*.

A combination of LABA and ICS is commonly recommended for asthma patients. Especially, ICS is known for its distinct inhibition of pulmonary inflammation (Barnes, 2010). In fact, our experiments with Formoterol and Budesonide (ICS) combination treatment on murine models demonstrated a significant reduction of both Th2 and Th17 responses. The result suggests that the significant role of Budesonide in preventing Formoterol's side effect. Also, our observation on the recovery of inflammation after stopping Budesonide for one month confirmed the Budesonide's transient effect in allergic asthma. Thus, the Budesonide's transient effect emphasizes that there is a need to adjust the frequency of administration on asthmatic patients to maintain the constant anti-inflammation effect.

From these results, we have supported that LABA treatment stimulates neutrophilic asthma in the lung, and the LABA-ICS combination is essential to prevent provoking unintended inflammation. In fact, our findings illustrate an interesting aspect that prevailing asthma treatments have a high potential of stimulating more severe inflammation. Especially, LABAs' side-effect in the airway suggests the necessity of a new therapeutic approach where β2AR agonists selectively act on ASM instead of DCs.

3.2 Further studies

In this study, we were able to find the pattern that LABAs inhibit *IRF4* expression in all three human DC-like monocyte cell lines. This result indicates that LABAs' ability to modulate DCs' cAMP signaling pathway to activate Th17 response. However, further experiments are needed in actual human DC cell lines since it was tested only through monocyte cell lines. Although LABAs themselves activate Th17 response through the cAMP signaling pathway, we

have confirmed that ICS can be a solution for this side-effect by maintaining LABAs' bronchodilation effect and preventing Th17 differentiation. However, we did not evaluate DCs' response to different bronchodilators' combination treatment. Recently, Long-acting muscarinic antagonists (LAMAs) with LABA combination treatment was discovered that they provide anti-inflammatory effect for COPD patients when it's treated with ICS (Milara et al., 2019). According to Milara et al.'s study, there was a distinct suppression of inflammation in neutrophils among chronic obstructive pulmonary disease (COPD) patients when LAMAs and LABAs were cotreated with ICS. This suggests that there is a possibility that LAMAs can prevent the increased Th17 response by LABAs in asthma. Thus, investigating the impact of LAMAs with LABAs on lung DCs might provide a solution for preventing the LABAs' side-effect. Additionally, LABA+ICS's effect on lung DCs has to be further tested in different concentrations to adjust the recommended dose of LABA+ICS in asthma treatment. Therefore, future experiments of different doses of LABA-ICS combination on DCs and investigating their effects on Th2 and Th17 differentiation will foster a better understanding of side-effects of common asthma drugs.

Materials and Method

Mice and Cells

C57B1/6J and OT2 mice were acquired from The Jackson Laboratory. Splenic OT2 $CD4^+$ T lymphocytes were isolated by EasySep™ Mouse $CD4^+$ T Cell Isolation Kit (StemCell Technologies). Bone Marrow (BM) cells were acquired from mice's bone marrow. For 7 days, BM cells were cultured with GM-CSF (10 ng/ml). Floating BM cells were harvested, and cDC2 BM cells ($CD11c^+CD135^+$) were isolated by using EasySep™ Mouse $CD11c^+$ Isolation Kit (StemCell Technologies). cDC2 BM cells were used for coculture with splenic OT2 $CD4^+$ T lymphocytes and for TFs analysis.

Lung resident DCs isolation

6 WT mice (C57B1/6J) were triggered with HDM (25 μg) to induce AHR. HDM is treated for 3 times a week for 2 weeks. On day 16, mice were sacrificed, and their lungs were collected and fragmented. HBSS with 20 μg/ml DNase, 0.5 mg/ml type 1A collagenase and 5% FBS were applied to the lung fragments. After 30 minutes of incubation, the lung cells were single-cell suspended through a 100 μm cell strainer. $CD103^+$ cDCs ($CD11c^+CD103^+$), $CD11b^+$ cDCs ($CD11c^+CD103^-CD11b^+CD64^-$), and MoDCs ($CD11c^+CD103^-CD11b^+CD64^+$) were isolated by FACS sorting and were used for TFs analysis.

Human DC-like monocytes cell lines

MUTZ-3 is a human myeloid leukemia cell line, which was acquired from Dr. Martin L. Yarmush (Rutgers University, New Jersey). a-MEM (Invitrogen, Carlsbad, CA, USA)

supplemented with 20% Fetal bovine serum, 50 mM b-mercaptoethanol and 10% 5637 cell-conditioned media was used for culturing MUTZ-3. MUTZ-3 was differentiated into DC-like cells under the growth media with GM-CSF (100 ng/ml, BioLegend, San Diego), TGF-b (10 ng/ml, R and D systems) and TNF-a (2.5 ng/ ml, R and D systems) for 7 days. THP-1 is a human leukemia cell line, which was acquired from ATCC. RPMI 1640 with 10% FCS was used for culturing THP-1 at a concentration of $2x10^5$ cells/ml. THP-1 was differentiated into DC-like cells with additions of rhIL-4 (100 ng/ml) and rhGM-CSF (100 ng/ml). HL-60 is a human leukemia cell line, which was acquired from ATCC. RPMI 1640 with 10% FCS was used for culturing HL-60 at a concentration of $2x10^5$ cells/ml. HL-60 was differentiated into DC-like cells in the culture media with calcium ionophore A23187 (180 ng/ml) and rhGM-CSF (100 ng/ml) for 24 hours.

Flow cytometry and intracellular staining

After samples were single-celled, FACS buffer (PBS with 2% FCS) was used in washing the samples. After the cells had been treated with appropriate antibodies, they were left in a incubator for 30 minutes. The cells were re-washed with the FACS buffer for two more times, and they were loaded on C6 Accuri flow cytometer from BD Biosciences and analyzed through FlowJo Software.

Reagents

OVA was obtained from Worthington Biochemical and GenScript. ELISA and FACS antibodies were purchased from eBiosciences, Invitrogen, and BD Pharmingen. HDM was

obtained from Greer. Anti-CD3 and antio-CD28 were purchased from BioXcell. Formoterol and CE3F4 were acquired from Tocris. Forskolin was purchased from Sigma-Aldrich.

Co-culturing of DCs and OT2 cells

BM cells were obtained from mice's femur and tibia bone marrow. GM-CSF (10ng/ml) were treated to these BM cells for 7 days. BMDCs were isolated from the culture by using EasySep^{TM} Mouse CD11c^{+} Isolation Kit (Stem Cell Technologies). Selected BMDCs were left one hour with OVA (100 μg/mL). After BMDCs were administered with treatments, they were incubated for one day in RPMI 1640 medium with 10% FCS at 37°C. Subsequently, BMDCs are moved to new medium and cocultured with OT2 CD4^{+} T lymphocytes for 3 days. For BMDCs, 0.5x10^{6} cells of BMDCs were cocultured with 0.5 0.5x10^{6} OT2 cells. For lung resident DCs, 0.3x10^{6} cells were cocultured with 0.15x10^{6} OT2 cells. After 3 days of coculture, effector OT2 T lymphocytes were isolated and restimulated with anti-CD3/anti-CD28 antibodies. 24 hours later, supernatants of restimulated OT2 cells were collected for cytokine profiling.

ELISA measurement of cytokines

The supernatants of cocultured helper T lymphocytes were harvested and sampled for ELISA of IL-4, IL-17A, and IFNγ cytokines. We used eBioscience's ELISA kit and followed its protocol for the cytokine profiling.

Quantitative PCR analysis

To obtain cDNA for each samples, we used RNA purification Kit from Thermo Fisher Scientific for RNA isolation. We used isolated RNA to reverse-transcribe cDNA with

Superscript III First-Strand system from Invitrogen. SYBR Green PCR Master Mix from Thermo Fisher Scientific were used to perform qPCR on cDNA. We used GAPDH to normalize the qPCR results. Followings are primer sequences that we used:

Irf4: F-AGATTCCAGGTGACTCTGTG, R-CTGCCCTGTCAGAGTATTTC,

Klf4: F-CTGAACAGCAGGGACTGTCA, R-GTGTGGGTGGCTGTTCTTTT,

Irf8: F-CGCTGTAGGAAAAGCAGACC, R-CCTCCAACAACACAGGGAGT,

Crem: F-GCTGAGGCTGATGAAAAACA, R-GCCACACGATTTTCAAGACA,

Muc5AC: F-TGGAGTCAGCACGAAAACAG, R-GCACTGGGAAGTCAGTGTCA

cAMP assay

ASM, BMDCs, and human DC-like cell lines were treated with Formoterol or forskolin (Fsk) and cultured in RPMI 1640 medium with 10% FCS at 37°C for 30 minutes. The medium was aspirated, and the cells were treated with 7.5% trichloroacetic acid (50 μl/ 1million cells). cAMP was normalized and analyzed by following Cayman Chemical's instructions.

Chronic asthma models and analysis

WT mice (C57BL/6J) were HDM-sensitized (25 μg) on day 0 and day 14 to induce HDM-derived asthma. Additionally, the asthma models were challenged by HDM (12.5 μg) or HDM (12.5 μg) with Formoterol (5 μg) for 2 times a week for 8 weeks. In Formoterol-budesonide experiments, the asthma models were challenged by HDM (12.5 μg), HDM (12.5 μg) with Formoterol (5 μg), or HDM (12.5 μg) with Formoterol (5 μg) and Budesonide (85 μg). On day 70, the asthma models were re-exposed to HDM (12.5 μg). On day 71, the airway hyperresponsiveness of the models were assessed through Methacholine challenge test.

Subsequently, cellular compositions of bronchoalveolar lavage were analyzed. Lung cells were single-cell suspended for the cytokine profiling. H&E, blue-Trichrome, and red-purple-PAS were used for staining lung tissues for historical analysis.

Statistical analysis

GraphPad Prism software is used for figures. Student's t-tests are used for data with 2 groups. ANOVAs are used for multiple groups.

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