UNIVERSITY OF CALIFORNIA SAN DIEGO

# cAMP in dendritic cell alters the fate of Th2 and Th17 responses

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Biology

by

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ABSTRACT OF THE THESIS

**cAMP in dendritic cell alters the fate of Th2 and Th17 responses**

by

Jun Hwan Kim

Master of Science in Biology

University of California San Diego, 2018

Professor Eyal Raz, Chair

Professor Elina Zuniga, Co-Chair

Dendritic cells are professional antigen presenting cells that activate naïve T cell to promote specific T cell responses. Until recently, the DCs' recognition of microbial or inflammatory stimuli through PRRs was solely recognized to initiate DCs' maturation. On the other hand, the role of PRR-independent activation of DCs has remained largely unexplored. This thesis will focus on a recent discovery of cAMP dependent DCs' maturation. We treated various types of DCs with GPCR ligands and other regulators affecting GPCR/cAMP pathway. Here, we show that DCs' varying levels of cAMP determine DCs' ability to promote either Th2 responses or Th17 responses. We discovered that the increased cAMP level reduced the expression of DC's transcription factors like IRF4 and KLF4. Subsequently, DC's increased cAMP level reduced Th2 responses and increased Th17 responses. In fact, this cAMP dependent DC maturation is so robust that inducing cAMP signaling alone allowed Th2-promoting DC to switch into Th17-promoting DC. Surprisingly, we discovered that DC's expression of IRF5 and inhibition of IRF4 are essential in Th17 polarization. In addition, we also described the implication of GPCR/cAMP pathway's role in the onset of neutrophilic asthma. Th2 response in the airway is known to promote eosinophilic asthma while additional Th17 response induce neutrophilic asthma. Here, we show that a long-term

exposure to asthma's common bronchodilator, LABA, also switches Th2 mediated eosinophilic asthma into Th17 mediated neutrophilic asthma by elevating DC's cAMP level. These findings delineate an unforeseen contribution of cAMP signaling in the regulation of innate and adaptive immunity.

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**Chapter 1**

# INTRODUCTION

## 1.1 Dendritic cells

Dendritic cells (DCs) are professional antigen presenting cells that have a remarkable capacity to present foreign antigens[1]. When immature DCs which reside in various tissues capture foreign antigen, these DCs uptake and process the antigen. In the meantime, inflammatory stimuli and microbial products initiate DCs' maturation which upregulate DCs immunogenic ability [2]. Upon maturation, DCs migrate to draining lymph nodes, where they present processed antigen to activate naïve T lymphocytes[2, 3]. Accordingly, DC is a key player in host defense system, bridging innate and adaptive immunity.

Since its discovery in 1978, diverse DC populations with unique immunogenic functions have been distinguished[4]. Based on different expressions of surface markers, DC's have been traditionally classified into several subsets, which comprise plasmacytoid DCs (pDCs), conventional DCs (cDCs) and monocyte-derived DCs (MoDCs)[1, 4, 45]. Namely, pDCs which are specialized in producing type I interferons express Siglec-H and Bst2[1, 4]. cDC is known to express CD11c[1]. Recently, cDC subsets have been further divided into 2 subtypes: cDC1 and cDC2[1]. cDC1s uniquely express CD103 while cDC2s express CD11b[1]. In addition, cDC1 stimulates T helper 1 cell (Th1) response from $CD4^+$ T cells and cytotoxic T cells (CTL) responses from $CD8^+$ T cells[5]. cDC2 promotes Th2 and Th17 response from $CD4^+$ T cells[5]. These two cDC subtypes also express a unique combination of transcriptional factors, further defining their identity. cDC1 express interferon regulatory factor 8 (IRF 8) [5, 6, 7]. cDC2's expression of IRF4 and Kruppel-like factor 4 (KLF4) promote Th2 polarization [5, 6, 7, 8]. Alternatively, cDC2's expression of NOTCH2 has been associated with Th17 response [7, 9]. MoDC is a unique DC subset that are derived from circulating monocytes exclusively during inflammation [45]. Monocyte's expression of Csf-1 is important for its differentiation into MoDC [48]. Namely, lung MoDC is known to express CD11b and CD11c like cDC2. MoDC has been shown various roles in inflammatory response, including functions that were attributed to cDC subsets. Yet, lung MoDC can be distinguished from cDC subset, by their expression of surface marker CD64 [45] (Fig. 1.1). Likewise, understanding what biases DCs to prime specific T cell response is an active area of research that is fundamental to understanding the behaviors of adaptive immune systems.

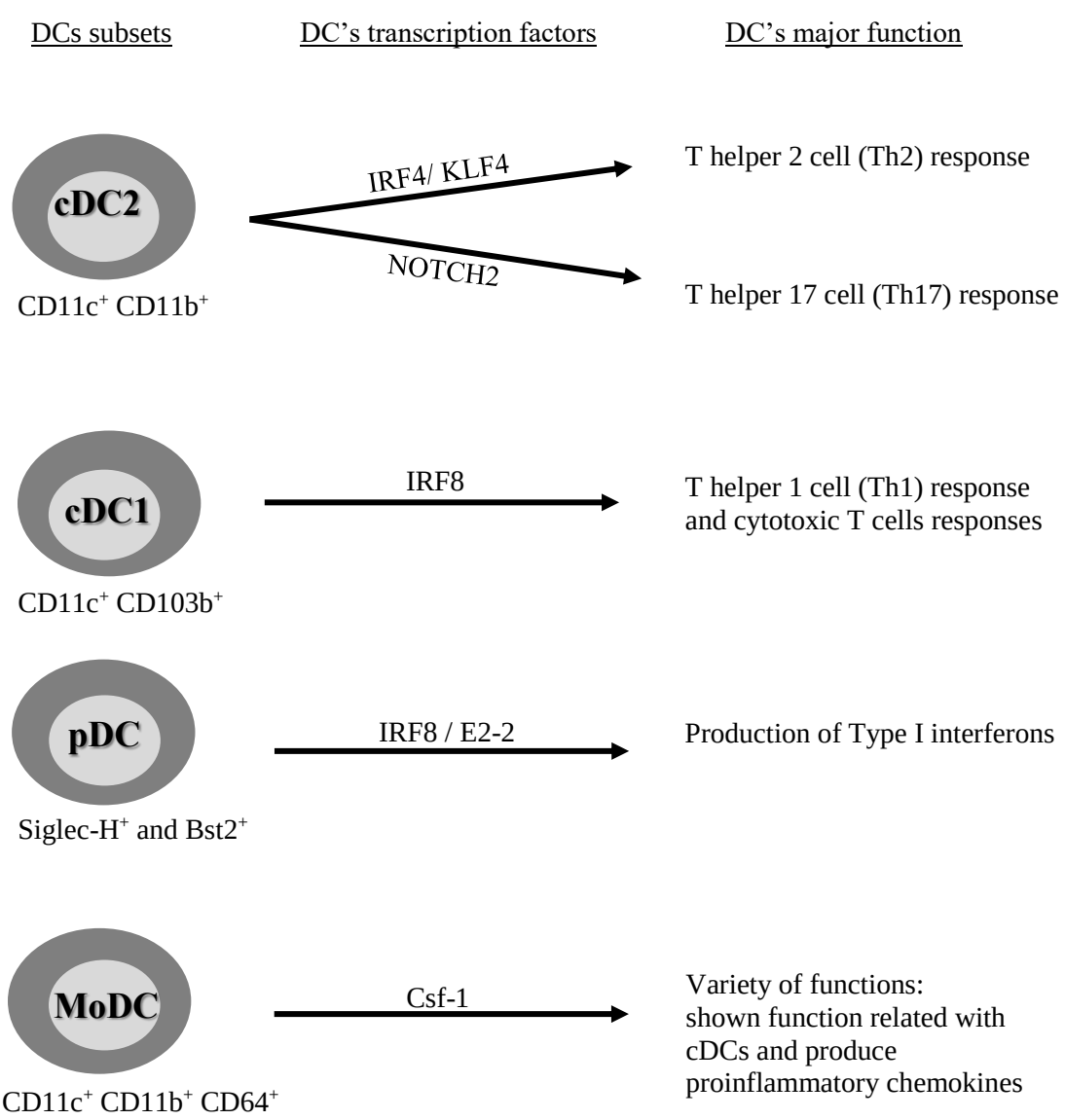


**Figure 1.1 Dendritic cell's classification**

Schematic illustration of DC's classification (based on surface markers), their important transcriptional factors, and their reported immunogenic functions. cDC2 can promote either Th2 or Th17 responses. cDC1 is specialized in promoting Th1 and cytotoxic T cell responses. pDC is known for its production of type I interferons. MoDC performs variety of functions during inflammations.

### 1.2 Pattern recognition receptors activating dendritic cells

DCs' pattern recognition receptors (PRRs) have been recognized to play a major role in DC's ability to elicit specific T cell response. PRRs are a set of germ line encoded receptors that are expressed on various innate immune cells, including DCs [10]. PRRs sense various dangers to the body and initiate a cascade of responses within DCs. This cascade of responses activates innate immunity and eventually shapes adaptive immunity. For instances, some classes of PPRs recognize damage-associated molecular pattern (DAMPs). PRRs can also sense conserved microbial and pathogenic structures like microbe-associated molecular pattern (MAMPs) and pathogen-associated molecular patterns (PAMPs) [2, 10, 11]. And recognition of these molecules associated with pathogen results in specific T cell responses. Namely, curdlan, a β-glucan, is a bacterial PAMP that is recognized by dectin-1, PRR, to elicit Th17 response [34]. Despite our expanding knowledge of PPRs' central role in DC reprogramming, the role of PRR-independent activation of DCs has remained largely unexplored. One particularly intriguing group of non-pattern recognition receptors is the G protein-coupled receptors (GPCRs). GPCR has been suggested to play some role in DC's immune responses [14, 15]. However, the extent of GPCR's role in DC's maturation remains elusive.

### 1.3 G-protein-coupled receptors

GPCRs are the largest family of transmembrane proteins in vertebrate that partake in an immense array of functions, including immune response. Various cells including DCs express GPCRs[14, 15]. Moreover, GPCRs can also recognize and bind to variety of signaling molecules like hormone peptides and lipids. Despite this stunning diversity, GPCRs share a conserved architecture and a common signaling mechanism [16, 17, 18]. Following its ligand binding, GPCRs activate heterotrimeric G proteins (αβγ) in the plasma membrane. Upon activation, the alpha subunit of the G protein exchange GDP for GTP. Then, $G_{\alpha}$ subunit dissociates from $G_{\beta\gamma}$ subunit to modulate other effector enzymes such as adenylyl cyclase (AC). Yet, there are subclasses within $G_{\alpha}$ subunit that regulate AC differently. $G_{\alpha s}$ activates AC to generate the second messenger, cAMP. Conversely, $G_{\alpha i}$ induces inhibition of AC, decreasing cAMP level. Next, cAMP can interact with intracellular cAMP receptors like protein kinase A (PKA) and exchange proteins of activated cAMP (Epac). Active PKA can phosphorylate diverse substrates like enzymes and transcriptional factors. Active Epac triggers activity of the Ras superfamily. The cell regulates this intricate activity of cAMP by phosphodiesterase (PDE). PDE hampers cAMP's activity by converting it into 5'-AMP. Since its discovery, GPCRs have stirred a great interest among scientist as nearly half of the current therapeutic drug in the market targets GPCRs[16, 17, 18, 19].

### 1.4 Asthma

Asthma, a heterogeneous disease with varying symptoms and severities, affects 300 million people globally. Allergic asthma is commonly characterized by airway inflammation, bronchial hyper-responsiveness and smooth-muscle remodeling. The patients' airways constrict upon the onset of inflammation, making them difficult to breathe. In severe cases of asthma, the cells in the airway produce thick and sticky mucus that block the airway. Unfortunately, uncontrolled severe asthma even leads to deaths [20, 21, 22, 23]. Based on specific T helper cell's influence on the airway, allergic asthma has been traditionally classified into 2 endotypes, which comprise Th2 mediated eosinophilic and Th17 mediated neutrophilic asthma. Various DC populations like pDCs, moDCs and cDCs reside in lung. Current discovery suggests cDC2 subtype to be responsible for shaping specific asthmatic endotypes [25]. cDC2 population that promotes Th2 response develops eosinophilic asthma. For instances, Th2 cells release cytokines to recruit eosinophils and promote inflammation. Moreover, Th2 cells promote B cells to produce IgE. Generally, eosinophilic asthma is characterized by mild symptoms. On the other hand, additional Th17 priming cDC2 population contributes to neutrophilic asthma. Th17 cells release cytokines to recruit neutrophils and promote inflammation. Moreover, Th17 cells promote B cells to heighten IgG1 secretion. Generally, neutrophilic asthma is characterized by moderate to severe symptoms. A mix of Th2 and Th17 immunity is known to cause neutrophilic asthma [23, 24, 25]. Yet, the molecular mechanism behind how cDC2 to promote either Th2 and Th17 response remains unclear. Moreover, the mechanism behind Th2-promoting DC to switch into Th17-promoting DC *in vivo* is not described as well. Furthermore, the conversion from eosinophilic asthma to neutrophilic asthma has not been fully studied *in vivo*.

Despite asthma's heterogeneity, the current treatments for these endotypes are rather undistinguished. Anti-inflammatory drug and bronchodilator are generally employed to ameliorate asthmatic symptoms. However, these treatments also come with serious side effects[25, 26]. Namely, β2 adrenoreceptor (β2AR) agonists like long-acting beta-agonists (LABAs) are typical bronchodilators to treat both asthma endotypes. A long-term and excessive use of LABA often results in exacerbation of the asthmatic symptoms which can lead to asthmatic related mortality [26]. To make the matters worse, neutrophilic asthma generally displays severe symptoms and resistance to the current treatment[24]. As β2AR also belongs to GPCR, β2AR agonists have been shown to activate AC and generate cAMP in airway smooth muscle cells; the activation of this GPCR pathway mediates bronchodilation[26]. Yet, the LABA's potential role of activating DC's GPCR/cAMP pathway to promote asthma has not been addressed.

### 1.5 Aims and Objective

This thesis will focus on a recent discovery of GPCR/cAMP pathway's novel role in reprogramming DCs. From our previous studies, DC's cAMP level is shown to be involved in promoting either Th2 or Th17 response. Yet the DC's underlying molecular mechanism behind this cAMP dependent DC maturation is still a mystery. Moreover, DC's transcriptional reprogramming during cAMP dependent DC maturation needs to be addressed.

In chapter 2, we verify that decreasing cAMP level in DCs lead to T helper 2 differentiation while elevating cAMP level in DCs lead to T helper 17 differentiation. We also identified a potential downstream effector protein for cAMP signaling. The DC's potential transcriptional regulation to induce Th17 response during cAMP dependent DC maturation is also shown. We also generated a $Gnas^{\Delta CD11c}$ mice to explore the switch from Th2-promoting DC to Th17-promoting DC; $Gnas^{\Delta CD11c}$ mice has $CD11c^{+}$ cell specific deletion of Gs alpha subunit. This leads to a lowered production of cAMP in its cDC2 population, promoting Th2 response.

In chapter 3, we investigated whether this GPCR/cAMP pathway mediated DC reprogramming is applicable *in vivo*. We induced allergic asthma in our animal model using either OVA/alum or HDM. Then, we further induced neutrophilic asthma by applying an additional LABA treatment. Adoptive transfers of LABA treated and HDM loaded BMDCs are also performed to verify that DCs alone can facilitate the onset neutrophilic asthma. We also seek to identify potential lung resident DCs that might influence the course of neutrophilic asthma via cAMP dependent DC's maturation. Ultimately, we hope to delineate a novel role of GPCR/cAMP signaling in innate and adaptive immunity from our studies.

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**Chapter 2.**

**RESULT- cAMP in dendritic cell determines the fate of Th2 and Th17 responses**

## 2.1. Introduction

Recently, the varying level of cAMP has been suggested to influence bone marrow derived APC ($CD11c^+$ BM-APC)'s ability to prime either Th2 or Th17 response. BM-APC from *Gnas*$^{\Delta CD11c}$ mice which express a low level of cAMP primes Th2 differentiation; the $CD11c^+$ DCs from *Gnas*$^{\Delta CD11c}$ mice have a deletion of GPCR's Gs alpha subunit, leading to its lowered cAMP expression[27]. Another recent study has shown that $CD11c^+$ DCs treated with cAMP elevating molecules like cholera toxin promoted Th17 differentiation[28]. Consequently, DCs' cAMP is suspected to have a crucial role in driving these two T helper cell subsets. Yet, we believed it is also crucial to validate that this cAMP mediated DC reprogramming occurred in the absence of PRR dependent signaling. Moreover, the underlying mechanism behind this cDC2 dependent Th2/Th17 bias remains poorly characterized. Furthermore, the transcription reprogramming responsible for Th2-promoting DC to switch into Th17-promoting DC is yet to be described. The following studies are performed in a hope to answer these questions.

## 2.2. cAMP signaling in DCs promotes Th17 response and transcription factor reprogramming

cDC1s, cDC2s and pDCs reside in spleen[1, 29]. Since cDC2 is specialized to prime Th2 and Th17 response from $CD4^+$ T cells, cDC2 population is used to investigate the mechanism behind Th2 and Th17 differentiation. Splenic cDC2s ($CD11c^+CD11b^+CD8^-$) are obtained from WT mice, through FACS sorting. Subsequently, splenic cDC2s are treated with cell permeable cAMP analog 8-(4-Chlorophenylthio) adenosine 3',5'-cyclic monophosphate (CPT) to simulate cDC2s' elevated cAMP level.[5] Concomitantly, isolated cDC2s are pulsed overnight with ovalbumin (OVA). Meanwhile, $CD4^+$ T cells are harvested from the spleen of OT-2 mice. Afterwards, $CD4^+$ T cells are co-cultured with OVA-pulsed splenic cDC2s. The resulting, activated CD4 T cells' supernatant are analyzed with ELISA for cytokine profiling. By comparing the levels of secreted Th-2 signature cytokine (IL-4) and Th-17 signature cytokine (IL-17a), cDC2s' ability to prime Th cell response has been assessed [3,7]. When compared to the untreated control, the OT-2 cells which are co-cultured with CPT treated cDC2 released less IL-4 (Fig. 2.2.1a) and more IL-17a (Fig. 2.2.1b). The expression of T cell lineage TF from these resultant OT-2 cells has been analyzed; Th1, Th2, Th17 and Treg cells express T-bet, GATA-3, RORγt and Foxp3, respectively [3, 12]. In fact, CPT treated group revealed a greater expression of RORγt and a lesser expression of GATA-3 than that of the untreated controlled group (Fig. 2.2.1c). This suggest that CPT treatment in DCs have

provoked Th17 response. Afterward, various DC reprogramming TFs have been assessed because we wanted to understand how DCs' expression of TFs are affected by cAMP singling; we seek to address DCs' TFs regulation that is responsible for Th17 differentiation. In addition, Crem was measured to evaluate the induction of cAMP signaling. Surprisingly, after CPT treatment, the level of cDC2's Th2 priming TFs like IRF4 and KLF4 decreased in a time-dependent manner. Moreover, CPT group showed a ten-fold higher Crem expression, suggesting that CPT successfully simulated cAMP signaling. The IRF8 levels are comparable between the CPT group and the untreated group. The untreated control group showed relatively unchanged expression of these TFs. (Fig. 2.2.1c). This suggest that cDC2 downregulates IRF4 and KLF4 when it acquires its Th17-promoting phenotype.

Since *Gnas*$^{\Delta CD11c}$ mice's cDC2 are missing its GPCR's Gs alpha subunit, cDC2's ability to induced cAMP signaling would be innately hampered [27]. Using this genetic model, we seek to confirm the role of cAMP-signaling in DC's maturation. As previously shown, spleen derived cDC2 ($CD11c^{+}CD11b^{+}CD8^{-}$) are obtained from *Gnas*$^{\Delta CD11c}$ mice and *Gnas* $^{fl/fl}$ mice. Similarly, these cDC2s are pulsed with OVA and treated with CPT. Subsequently, the cDC2s from the 2 groups are co-cultured with splenic OT2 CD4+ T cells. When compared to the *Gnas* $^{fl/fl}$ group without the treatment, the *Gnas*$^{\Delta CD11c}$ group without CPT treatment showed an elevated level of IL-4 and reduced level of IL-17a, (Fig. 2.2.2a and 2.2.2b). This suggests that *Gnas*$^{\Delta CD11c}$ mice favor Th2 response over Th17 response innately due to our genetic modification. However, upon CPT treatment, both groups showed drastically reduced IL-4 secretion and increased IL-17a secretions (Fig. 2.2.2a and 2.2.2b). Moreover, despite *Gnas*$^{\Delta CD11c}$ mice's predisposed bias toward Th2 response, its IL-4 and IL-17a levels are comparable to that of the fl/fl after the CPT treatment (Fig. 2.2.2a and 2.2.2b). Again, the OT-2 cells' expressions of T cell lineage TFs have been analyzed to verify Th17 biased response of the CPT treated groups. Before CPT treatment, *Gnas*$^{\Delta CD11c}$ group exhibits a greater induction GATA-3 compared to that of the untreated fl/fl group (Fig. 2.2.2c). Yet, after CPT treatment, *Gnas*$^{\Delta CD11c}$ group reduced its expression of GATA-3 (Fig. 2.2.2c). Moreover, both *Gnas* $^{fl/fl}$ and *Gnas*$^{\Delta CD11c}$ group upregulated RORγt upon CPT treatment (Fig. 2.2.2c). Foxp3 and T-bet inductions remain unchanged by the CPT treatment (Fig. 2.2.2c). This suggest that CPT treatment alone prevented predisposed Th2 response in *Gnas*$^{\Delta CD11c}$ DC. Instead, *Gnas*$^{\Delta CD11c}$ DC is switched to promote Th17 response. Moreover, DC's TFs are measured to assess the effect of CPT onto DCs' transcriptional reprogramming. Initially, *Gnas*$^{\Delta CD11c}$ DCs express higher Th2 priming TFs like IRF4 and KLF4 than the fl/fl (Fig. 2.2.2d). This substantiate that *Gnas*$^{\Delta CD11c}$ DCs are prone to drive Th2 polarization. Yet, in a time dependent manner, CPT treatment lowers IRF4 and KLF4 levels for both groups (Fig. 2.2.2d). IRF8 activity remains unaltered for both groups but Crem expression increased after the addition of CPT (Fig. 2.2.2d). Together these results suggest that DCs' downregulation of IRF4

and KLF4 is associated with induction of Th17 differentiation. Hence, intracellular staining for IRF4 is executed for these groups to further substantiate above results. Before CPT treatment, *Gnas*$^{\Delta CD11c}$ DCs' IRF4 expression is 1.5-fold greater than that of the *Gnas* $^{fl/fl}$. Yet, the addition of CPT lowered IRF4 expression from the both (Fig. 2.2.2e). Cumulatively, cDC2s can be switched from Th2-promoting DC to Th17-promoting DC by inducing cAMP signaling. Moreover, DCs downregulate IRF4 and KLF4 when cAMP signaling occurs.

So far IRF4 and KLF4 are shown to be downregulated by cAMP signaling. However, the downstream targets of cAMP signal that modulate this TF reprogramming remain elusive. Moreover, we wanted to check whether bone marrow derived APCs (BM-APCs) also adopt this mechanism to polarize Th17 cells. Hence, BM-APCs ($CD11c^+CD135^+$) are obtained from WT mice. Subsequently, BM-APCs are treated with various cAMP elevating molecules and co-cultured with OT-2 $CD4^+$ T cells, as shown before. Upon the treatments, the BM-APCs also polarized Th17 response like splenic cDC2 (Fig. 2.2.3a and b). Again, T cell lineages TFs of the OT-2 cells have been analyzed to verify Th cell differentiation. The treatment group shows nearly 2-folds higher expression of RORγt than that of the untreated (Fig. 2.2.3c). The DC's elevated cAMP signal appears to induce Th17 immune responses. When DC's TFs are assessed, the treatment groups again lowered Th2 priming TFs like IRF4 and KLF4 in a time dependent manner (Fig. 2.2.3.d and e). Subsequently, we investigated the downstream targets of cAMP signal that meditate inhibition of IRF4 expression. PKA and Epac were the best candidates because they are known to be cAMP's major downstream effectors [30, 31]. BM-APCs are treated with $G_{as}$ agonist, PGE2, to elevate cAMP level which leads to Th17 response. BM-APCs are also treated with PKA or Epac inhibitors to ascertain which effector regulates the DC reprogramming. Like CPT treatment, the addition of PGE2 alone lead to downregulation of IRF4. Yet, PKA inhibitor, Rp-cAMP, significantly reverted the effect of PGE2, elevating IRF4 level (Fig. 2.2.3.f). However, Epac inhibitor, CE3F4, failed to yield a substantial change (Fig. 2.2.3.f). This result suggests that PKA is the downstream molecular targets of cAMP signal and responsible for downregulating IRF4. Since DC's Notch2 expression has a major role in Th17 differentiation, the expression of Notch2 receptor has been assessed in BMDC [32]. After BM-APCs are treated with cAMP elevating molecules, BMDCs expressed a higher level of Notch2 mRNA and Notch2 receptors than the untreated BMDCs (Fig. 2.2.3.g and h). This further substantiates that the DCs with heightened cAMP level exhibit Th17-biasing DCs' phenotype.

The BM APCs from *Gnas*$^{\Delta CD11c}$ mice is also examined to confirm that cAMP signaling can switch Th2-promoting DC to Th17-promoting DC. As shown previously, BM-APCs are obtained from *Gnas*$^{\Delta CD11c}$ mice and *Gnas*$^{fl/fl}$ mice. Similarly, these BM-APCs are pulsed with OVA and treated with or without CPT. Subsequently, the

BM-DCs from the two groups are co-cultured with OT2 $CD4^+$ T cells. When compared to the *Gnas*$^{fl/fl}$ group without treatment, the OT-2 cells from untreated Gnas KO group showed an elevated level of IL-4 (Fig. 2.2.4.a) and reduced level of IL-17a (Fig. 2.2.4.b). Similarly, upon CPT treatment, Gnas$^{\Delta CD11c}$ and *Gnas*$^{fl/fl}$ groups showed drastically reduced IL-4 secretion (Fig. 2.2.4.a) and increased IL-17a expression (Fig. 2.2.4.b). Again, the CPT treatment led to an unaltered production of IFNγ for both groups (Fig. 2.2.4.c). This suggest that CPT treatment alone prevented predisposed Th2 response in *Gnas*$^{\Delta CD11c}$ BMDC. Instead, *Gnas*$^{\Delta CD11c}$ BMDC is switched to promote Th17 response. To support this finding, the cytokine expression of $CD4^+$ T cells from IL-4 eGFP / OT2 and IL-17A eGFP / OT2 mice are observed; these $CD4^+$ T cells with reporter activity are cultured with BM-APCs from *Gnas*$^{\Delta CD11c}$ and *Gnas*$^{fl/fl}$ mice. Under FACS analysis, $CD4^+$ T cocultured with untreated *Gnas*$^{\Delta CD11c}$ BMDC express a higher level of IL-4 and lower level of IL-17a than the untreated fl/fl control group (Fig. 2.2.4.d and e). Yet, CPT treatment favored the production of Th17's signature cytokine over that of the Th2 for both groups (Fig. 2.2.4.d and e). Again, the OT-2 cells' expressions of T cell lineage TFs have been analyzed. Before CPT treatment, *Gnas*$^{\Delta CD11c}$ group exhibits a greater induction GATA-3 compared to that of the untreated fl/fl group (Fig. 2.2.4.f). Yet, after CPT treatment, *Gnas*$^{\Delta CD11c}$ group reduced its expression of GATA-3 (Fig. 2.2.4.f). Moreover, both *Gnas*$^{\Delta CD11c}$ and *Gnas*$^{fl/fl}$ groups' expression of RORγt are greatly increased by CPT treatment (Fig. 2.2.3.f). Foxp3 and T-bet induction is relatively unaffected by the CPT treatment (Fig. 2.2.4.f). Again, DC TFs are measured. As we have shown with splenic cDC2s, initially, BM-APCs from *Gnas*$^{\Delta CD11c}$ group upregulate IRF4 and KLF4 than that of the untreated fl/fl control (Fig. 2.2.4.g). Yet, in a time dependent manner, CPT treatment lowers IRF4 and KLF4 levels for both groups (Fig. 2.2.4.g). IRF8 activity remains unaltered for both groups while Crem is upregulated by CPT treatment (Fig. 2.2.4.g). This supports the notion that CPT treatment alone switched Th2-promoting *Gnas*$^{\Delta CD11c}$ BMDC to Th17-promoting BMDC. Moreover, BMDCs also downregulate IRF4 and KLF4 when it promotes Th17 differentiation.

Together, we showed that the activation of cAMP dependent pathway has an intimate role in shaping splenic cDC2 and BMDC' ability to prime Th17 response. Our data suggest that a low cAMP level in the DCs allows a specific set of transcription factors (IRF4 and KLF4) to be upregulated. These TFs are known to prime Th2 response. Yet, when DC's cAMP level is manually amplified by cAMP elevating molecules, IRF4 and KLF4 were downregulated. Then, these DCs with inhibited IRF4 activity promoted Th17 differentiation, preferentially. Furthermore, PKA is identified as the downstream target of cAMP that orchestrates inhibition of IRF4. In fact, this reprogramming via cAMP is so robust that it switched *Gnas*$^{\Delta CD11c}$ DCs from biasing Th2 response to inducing Th17 response, upon CPT treatment.

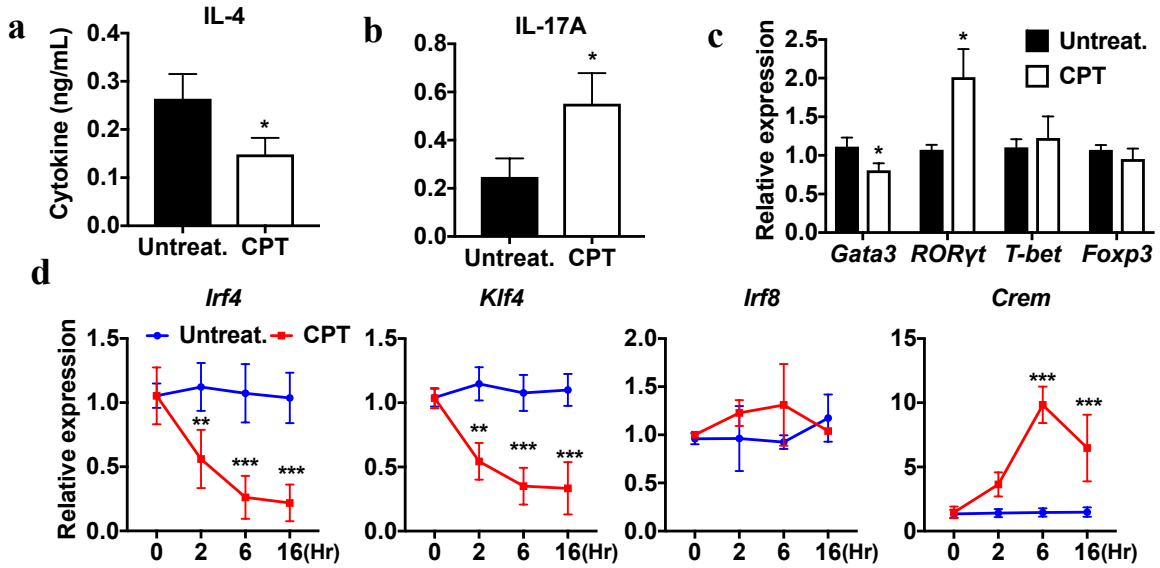



**Figure 2.2.1 cAMP signaling in cDC2 promotes Th17 response and transcription factor reprogramming**

**(a) and (b)** Cytokine levels (IL-4 and IL-17a) of splenic OT2 $CD4^+$ T cells which has been co-cultured with splenic cDC2 ($CD11c^+CD11b^+CD8^-$)from WT mice. These WT splenic cDC2s are either treated with or without CPT and loaded with OVA. The OT2 $CD4^+$ T cells have been stimulated with anti-CD3 and anti-CD28 antibodies. **(c)** the level of (mRNA) lineage commitment factors (Gata3, RORγt, T-bet, and Foxp3) have been assessed for isolated OT2 $CD4^+$ T cells through qPCR analysis. These T cells are co-cultured with WT cDC2s that have been either treated with or without CPT. **(d)** the level of (mRNA) DC TFs (Irf4, Klf4 Irf8 and Crem) have been assessed for isolated WT cDC2s through qPCR analysis. These cDC2s have been either treated with or without CPT. Two-way ANOVA with Sidak's multiple comparisons test has been applied; n=3 in each group, ** $p<0.01$, ***$p<0.001$. Effect of CPT treatment; Irf4 ($p=0.002$), Klf4 ($p<0.001$) and Crem ($p<0.001$).

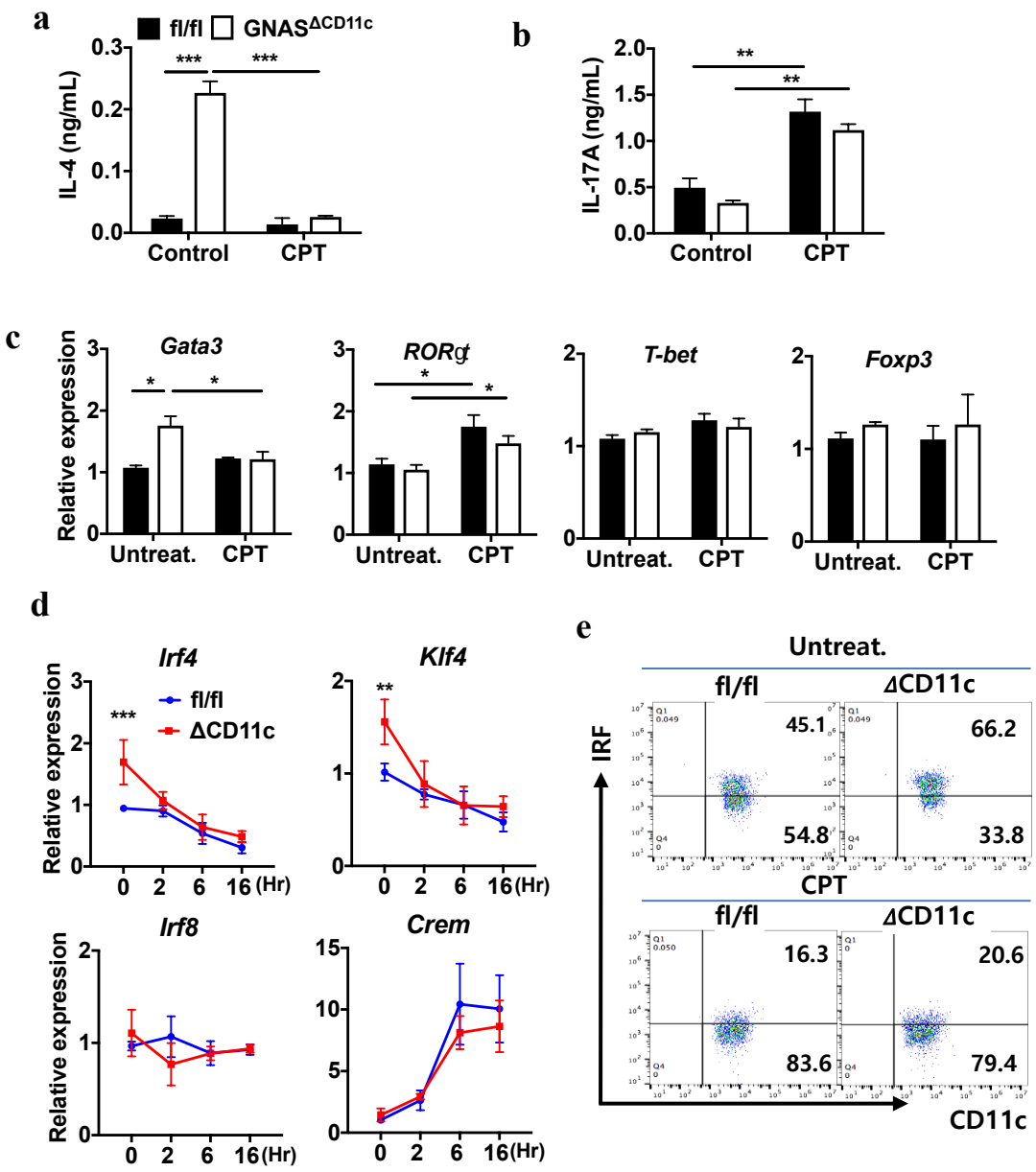
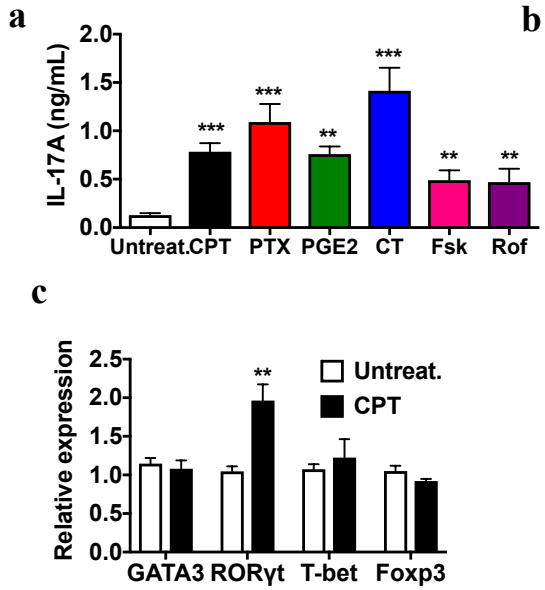



**Figure 2.2.2 cAMP signaling switches pro-Th2 cDC2 to a pro-Th17 cDC2**

**(a, b)** Cytokine levels (IL-4 and IL-17a) of splenic OT2 $CD4^+$ T cells which has been co-cultured with splenic cDC2 ($CD11c^+CD11b^+CD8^-$) from $Gnas^{\Delta CD11c}$ or $Gnas^{fl/fl}$ mice. These DCs are either treated with or without CPT and loaded with OVA. The OT2 $CD4^+$ T cells have been stimulated with anti-CD3 and anti-CD28 antibodies. **(c)** the level of (mRNA) lineage commitment factors (Gata3, RORγt, T-bet, and Foxp3) have been assessed for isolated OT2 $CD4^+$ T cells through qPCR analysis. These T cells are co-cultured with cDC2s from $Gnas^{\Delta CD11c}$ or $Gnas^{fl/fl}$ mice that have been either treated with or without CPT. **(d)** the level of (mRNA) DC TFs (Irf4, Klf4 Irf8 and Crem) have been assessed for isolated cDC2s through qPCR analysis. These cDC2s from $Gnas^{\Delta CD11c}$ or $Gnas^{fl/fl}$ mice have been either treated with or without CPT. Effect of CPT treatment; *Irf4* ($p$=0.001), *Klf4* ($p$<0.001) and *Crem* ($p$<0.001). **(e)** FACs analysis of splenic cDC2s from $Gnas^{\Delta CD11c}$ or $Gnas^{fl/fl}$ mice. These DCs are treated with CPT for 48hrs. Intracellular staining is used to assess IRF4 level upon CPT treatment. Two-way ANOVA with Sidak's multiple comparisons test has been applied; $n$=3 in each group, ** $p$<0.01, ***$p$<0.001.




b

| Cytokine (ng/ml) | Untreat. | CPT | *P value* |
|---|---|---|---|
| IL-4 | 0.099±0.023 | 0.054±0.021 | N.S |
| IL-5 | 0.443±0.091 | 0.337±0.039 | N.S |
| IL-10 | 1.1±0.067 | 0.875±0.068 | N.S |
| IFN-g | 0.573±0.093 | 0.834±0.088 | N.S |




**Figure 2.2.3 cAMP signaling in BMDC promotes Th17 response and transcription factor reprogramming**

**(a)** Cytokine levels (IL-17a) of splenic OT2 $CD4^+$ T cells which has been co-cultured with BM-APC from WT mice. These WT BMDCs are either treated with various cAMP elevating molecules: CPT, pertussis toxin (PTX), prostaglandin $E_2$ ($PGE_2$), cholera toxin (CT), forskolin (Fsk), and rolipram (Rol). The OT2 $CD4^+$ T cells have been stimulated with anti-CD3 and anti-CD28 antibodies. **(b)** Cytokine levels (IL-4, IL-5, IL-10 and IFNγ) of splenic OT2 $CD4^+$ T cells which has been co-cultured with BMDCs from WT mice. These WT BMDCs are either treated with or without CPT and loaded with OVA. The OT2 $CD4^+$ T cells have been stimulated with anti-CD3 and anti-CD28 antibodies. **(c)** the level of (mRNA) lineage commitment factors (Gata3, RORγt, T-bet, and Foxp3) have been assessed for isolated OT2 $CD4^+$ T cells through qPCR analysis. These T cells are co-cultured with WT BMDCs that have been either treated with or without CPT. Two-way ANOVA with Sidak's multiple comparisons test has been applied; $n$=3 in each group, different from untreated in the CPT-treated group; * $p$<0.05, ** $p$<0.01, *** $p$<0.001.

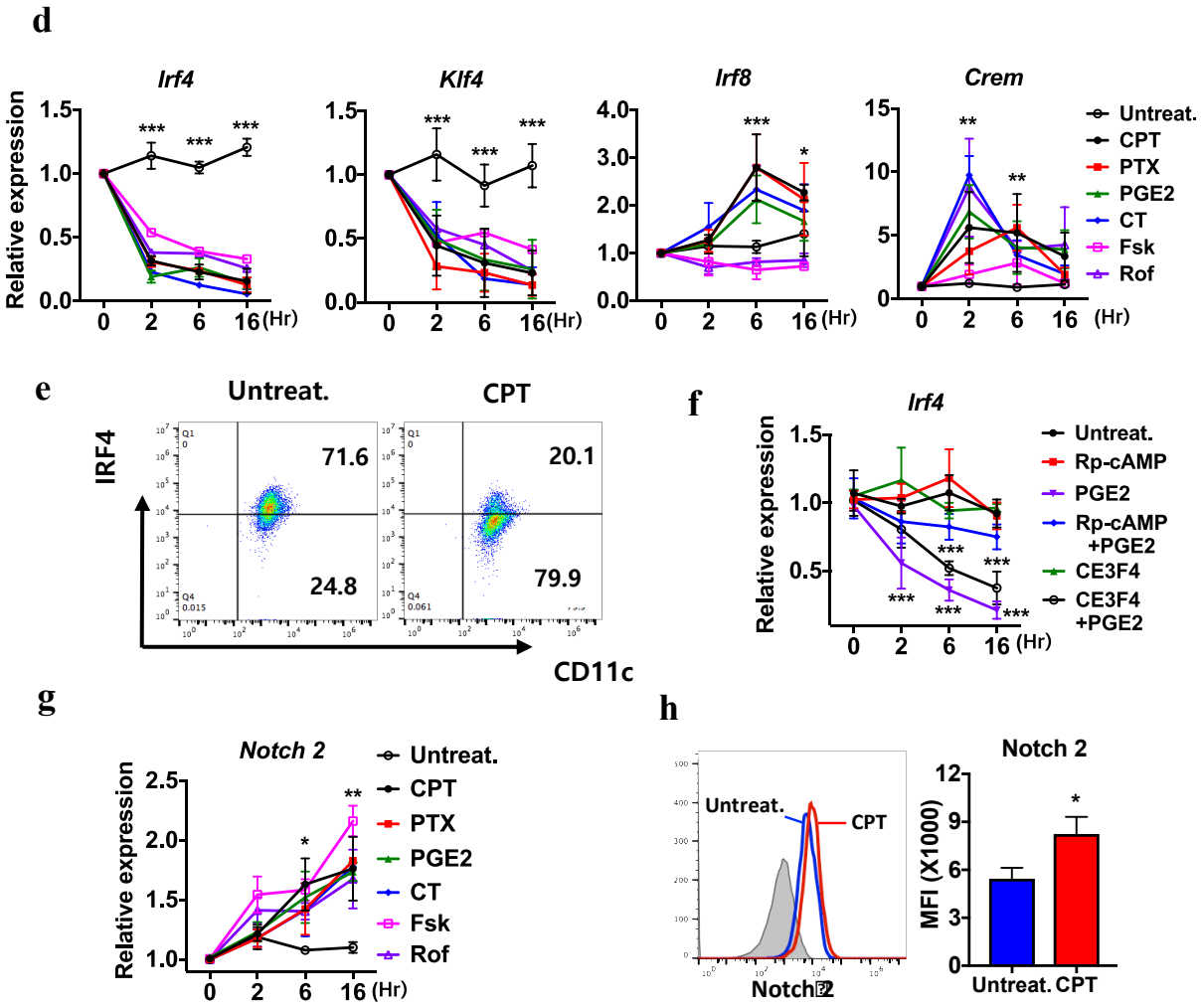



**Figure 2.2.3 cAMP signaling in BMDC promotes Th17 response and transcription factor reprogramming.**

**(d)** the level of (mRNA) DC TFs (Irf4, Klf4 Irf8 and Crem) have been assessed for isolated WT BMDCs through qPCR analysis. These BMDCs have been either treated with or without cAMP elevating molecules. Two-way ANOVA with Sidak's multiple comparisons test has been applied; $n$=3 in each group, different from untreated in the CPT-treated group; * $p$<0.05, ** $p$<0.01, ***$p$<0.001. Effect of treatment; *Irf4* ($p$<0.001), *Klf4* ($p$<0.001), *Irf8* ($p$<0.001) and *Crem* ($p$< 0.001). **(e)** FACs analysis of BMDCs from WT mice. These BMDCs are treated with or without CPT for 48hrs. Intracellular staining is used to assess IRF4 level upon CPT treatment. **(f)** the level of (mRNA) IRF4 have been assessed for isolated WT BMDCs through qPCR analysis. These BMDCs have been either treated with $PGE_2$ or $PGE_2$ and PKA inhibitors (Rp-cAMP) or $PGE_2$ and Epac inhibitor (CE3F5). **(g)** the expression of mRNA and **(h)** protein of Notch2 have been assessed for isolated WT BMDCs through qPCR analysis and FACs analysis. These DCs have been either treated with or without cAMP elevating molecules. Two-way ANOVA and Sidak's multiple comparisons test have been applied; $n$=3 in each group, different from untreated in the CPT-treated group;p<0.05, ** p<0.01. Data are mean ± s.e.m, n=3 in each group; * p<0.05, ** p<0.01, ***p<0.001.

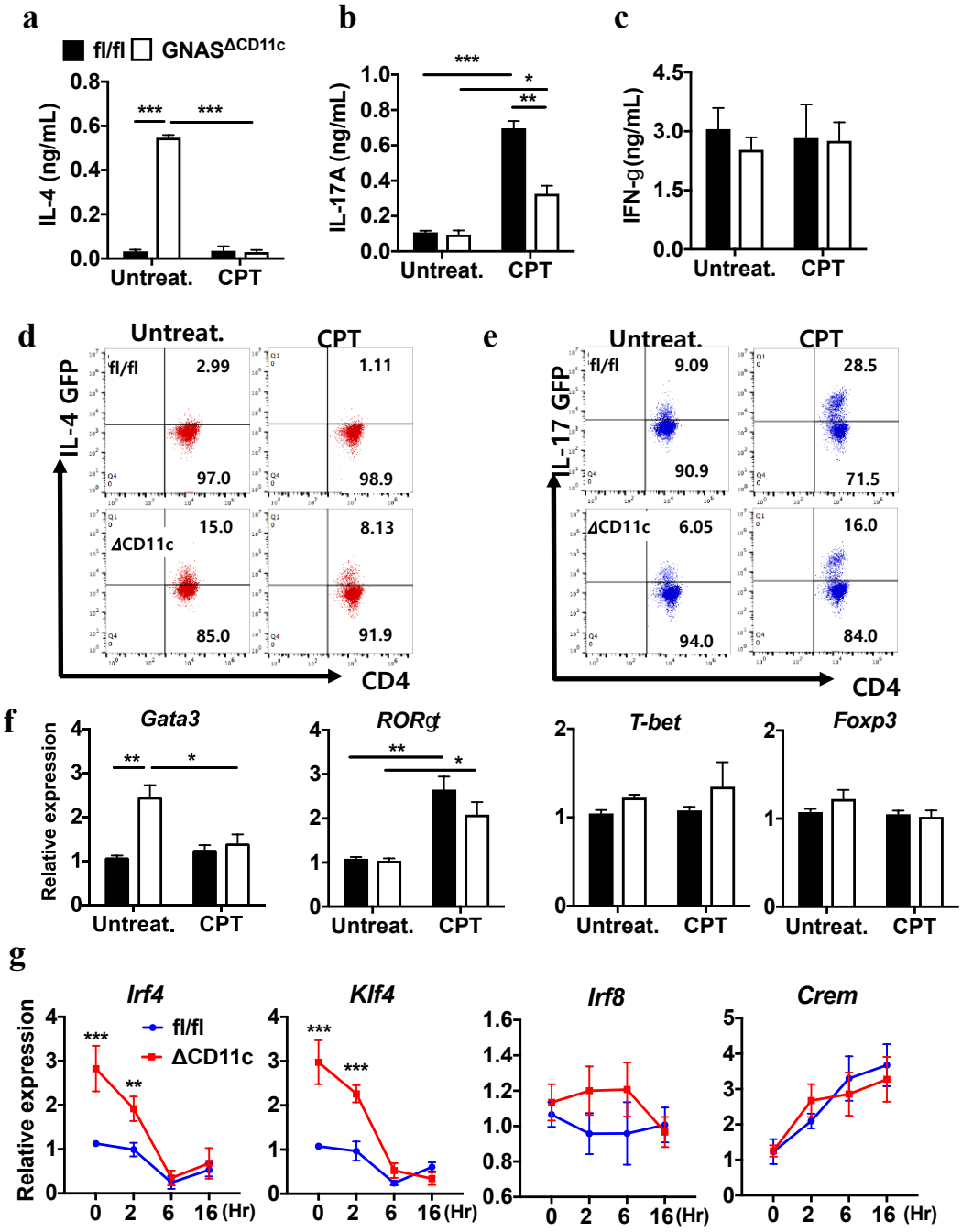



**Figure 2.2.4 cAMP signaling switches pro-Th2 BMDC to a pro-Th17 BMDC.**

**(a,b,c)** Using ELISA**,** Cytokine levels (IL-4, IL-17a and IFNγ) of splenic OT2 CD4$^+$ T cells are obtained. T cells have been co-cultured with BM-APCs from *Gnas*$^{\Delta CD11c}$ or *Gnas*$^{fl/fl}$ mice. These BMDCs are either treated with or without CPT and loaded with OVA. The OT2 CD4$^+$ T cells have been stimulated with anti-CD3 and anti-CD28 antibodies. **(d)** Naïve CD4$^+$ T cells from IL-4eGFP/OT2mice and **(e)** IL-17A eGFP/OT2 mice have been co-cultured with BM-APCs from *Gnas*$^{\Delta CD11c}$ or *Gnas*$^{fl/fl}$ mice. FACs analysis is performed on T cells that are stimulated with PMA and ionomycin. **(f)** the level of (mRNA) lineage commitment factors (Gata3, RORγt, T-bet, and Foxp3) have been assessed for isolated OT2 CD4$^+$ T cells through qPCR analysis. These T cells are co-cultured with BMDCs from *Gnas*$^{\Delta CD11c}$ or *Gnas*$^{fl/fl}$ mice that have been either treated with or without CPT. Two-way ANOVA and Sidak's multiple comparisons test have been applied. **(g)** the level of (mRNA) DC TFs (Irf4, Klf4 Irf8 and Crem) have been assessed for isolated *Gnas*$^{\Delta CD11c}$ or *Gnas*$^{fl/fl}$ BMDCs through qPCR analysis. These BMDCs have been treated with CPT. Two-way ANOVA with Sidak's multiple comparisons test has been applied; $n$=3 in each group, different from untreated in the CPT-treated group; ***$p$<0.001. Effect of CPT treatment; *Irf4* ($p$<0.001), *Klf4* ($p$<0.001) and *Crem* ($p$< 0.001). Effect of strain: *Irf4* ($p$< 0.001) and *Klf4* ($p$< 0.001). Data are mean ± s.e.m, $n$=3 in each group; * $p$<0.05, ** $p$<0.01, ***$p$<0.001.

### 2.3. Dectin-1-activated dendritic cells induce Th17 response and downregulate IRF4/KLF4

So far, mRNA expressions of IRF4 and KLF4 are consistently downregulated in Th17 biasing DCs with elevated cAMP level. We wanted to investigate whether this was a unique TF regulation in DCs with increase cAMP level, the PRR independent DC maturation, or a general regulation mediated by DC, including PRR dependent DC maturation, to drive Th17 response. Namely, Dectin-1 mediated DC activation is known to evoke Th17 response in the absence of cAMP signaling[33]. Dectin-1 is a c-type lectin that specifically recognize β-glucans such as curdlan[33,34,35,36]. Curdlan is a bacterial PAMP and a linear polymer of (1,3)- β-glucans which is produced by the soil bacterium, *Alcaligenes faecalis*[33,34,35,36]. Upon Dectin-1 activation, the Syk-CARD9 pathway is initiated to drive DCs to favor Th17 response[33]. Hence, curdlan mediated DC maturation allows detection of additional cases of IRF4/ KLF4 inhibition in Th17-biasing DCs while preventing IRF4 downregulation by cAMP signaling.

For these reasons, curdlan is applied to cDC2s to generate PRR dependent pro-Th17 DCs. As shown previously, the DCs are derived from the spleens or bone marrow of WT mice. Then, DCs are treated with curdlan and pulsed with OVA so that it can be co-cultured with OT-2 $CD4^+$ T cells. After the co-culture, OT-2 $CD4^+$ T cells from the curdlan treated group produced a higher level of IL-17a than that of the untreated control group (Fig. 2.3a and 2.3d). Upon curdlan treatment, the treatment group expressed a reduced level Gata3, but a higher level of RORγt (Fig. 2.3b and 2.3e). These data suggest that curdlan treated DCs successfully promoted Th17 response. Surprisingly, the curdlan treated splenic cDC2 and BMDC also expressed lower levels of IRF4 and KLF4 than that of the untreated control (Fig. 2.3c and 2.3f). This result suggests that curdlan treatment downregulates of IRF4 and KLF4 for DCs that now favor Th17 polarization. Crem activity is also lowered in a time dependent manner for the treatment group. This substantiate that the DC reprogramming via curdlan occurred without cAMP signaling (Fig. 2.3c and 2.3f). Both cAMP dependent and Dectin-1 dependent pathways ultimately inhibit IRF4 and KLF4 to drive Th17 response. To further characterize Dectin-1 mediated Th17 bias, PKA's role in Dectin-1 pathway is also investigated. Again, the T cells' IL-17a level is measured using ELISA. The OT-2 T cells from the curdlan and Rp-cAMP treated group, still expressed a higher level of IL-17a than the untreated group, suggesting that Dectin-1 mediated DC activation can occur under suppressed PKA activity (Fig. 2.3d).

Collectively, curdlan treated DCs are verified to prime Th17 response. This PPR dependent maturation occurred in the absence of cAMP and PKA activity. Yet, activated Dectin-1 pathway still downregulated DC's IRF4 and KLF4's mRNA expression. Our data suggest that downregulation of IRF4 and KLF4 is a common TF reprogramming for DCs, during their Th17 polarization.

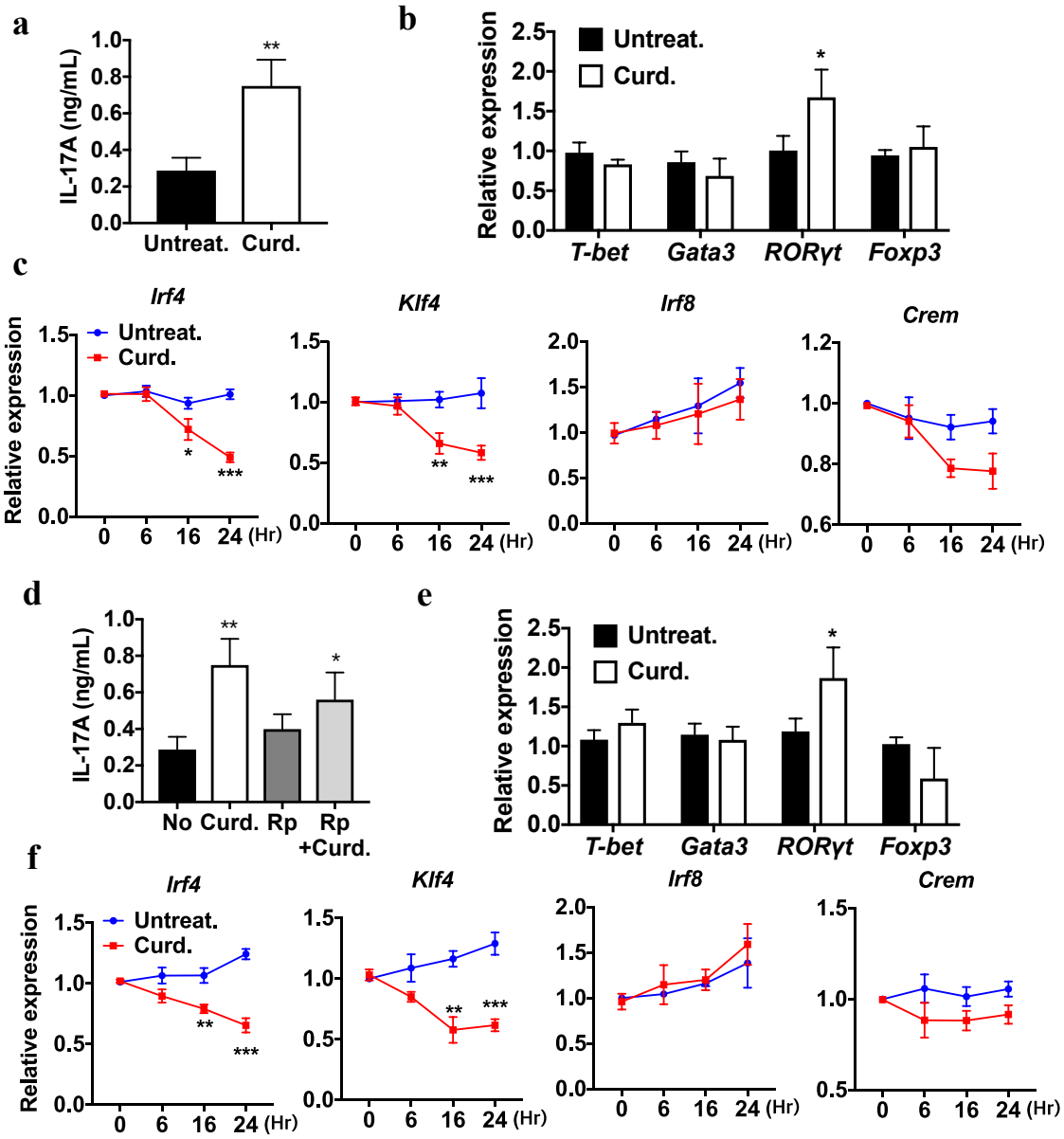



**Figure 2.3 Dectin-1-activated dendritic cells induce Th17 response and downregulate its IRF4/KLF4 level**

**(a)** Cytokine level (IL-17a) of splenic OT2 $CD4^+$ T cells which has been co-cultured with splenic cDC2 ($CD11c^+CD11b^+CD8^-$) from WT mice. These WT DCs are either treated with or without curdlan and loaded with OVA. The OT2 $CD4^+$ T cells have been stimulated with anti-CD3 and anti-CD28 antibodies. **(b)** the level of (mRNA) lineage commitment factors (Gata3, RORγt, T-bet, and Foxp3) have been assessed for isolated OT2 $CD4^+$ T cells through qPCR analysis. These T cells are co-cultured with WT DCs that have been either treated with or without curdlan. **(c)** the level of (mRNA) DC TFs (Irf4, Klf4 Irf8 and Crem) have been assessed for isolated WT DCs through qPCR analysis. These DCs have been either treated with or without curdlan. **(d)** Cytokine level (IL-17a) of splenic OT2 CD4+ T cells which has been co-cultured with BM-DCS from WT mice. These WT DCs are either treated with or without curdlan and loaded with OVA. The OT2 $CD4^+$ T cells have been stimulated with anti-CD3 and anti-CD28 antibodies. DCs are also treated with Rp-cAMP (PKA inhibitor) 6 hours before the curdlan treatment. **(e)** the level of (mRNA) lineage commitment factors (Gata3, RORγt, T-bet, and Foxp3) have been assessed for isolated OT2 CD4+ T cells through qPCR analysis. These T cells are co-cultured with WT BM-DCs that have been either treated with or without curdlan. **(f)** the level of (mRNA) DC TFs (Irf4, Klf4 Irf8 and Crem) have been assessed for isolated WT BM-DCs through qPCR analysis. These DCs have been either treated with or without curdlan. Two-way ANOVA with Sidak's multiple comparisons test has been applied; n=3 in each group, ** $p<0.01$, ***$p<0.001$

**2.4. The inhibition of IRF4 expression in DCs is essential in Th17 polarization**

IRF4 is an important TF for cDC2s; IRF4 expression promotes cDC2s' proper development and cDC2's migration to draining lymph nodes for eventual T cell priming [37, 38]. So far, our results consistently suggest that both cAMP dependent and Dectin-1 dependent DC's maturations downregulates their IRF4 expression. And the resulting DCs promoted Th17 response. Hence, we suspected IRF4 downregulation is essential in driving Th17 immunity. Yet, we still have not fully demonstrated the inhibited IRF4 expression's role in inducing Th17 differentiation. Thus, we investigated whether the deletion of IRF4 gene alone can evoke Th17 polarization. Two models have been implemented to answer this question: IRF4-inducible and *IRF4*$^{\Delta CD11c}$ mice models.

A tetracycline (Tet)-inducible system controlling IRF4 expression has been employed to address this question. *IRF4* $^{-/-}$ mice expressing a Tet -inducible allele of IRF4 via transcriptional activator (M2rtTA) has been engineered. These IRF4-inducible mice only express IRF4, upon a treatment with tetracycline analog, Doxycycline (Dox) [39]. This is verified using FACs analysis of the BM-APCs from IRF4-inducible mice. After Dox treatment, the treatment group expressed IRF4 3-folds higher than that of the untreated control (Fig. 2.4 a). With previously depicted methods, BM-APCs from IRF4 -inducible mice are co-cultured with OT2 $CD4^+$ T cells to measure the expression of IL-5 and IL-17a. The absence of IRF4 expression inhibited secretion of Th2 cytokine, IL-5 (Fig. 2.4 b), but encouraged secretion of IL-17a (Fig. 2.4c). Yet, when Dox is treated to IRF4 inducible DCs, we no longer saw this bias toward Th17 response (Fig. 2.4b and 2.4c). This suggest IRF4 inhibition alone phenocopied the effect of CPT treatment. When this co-culture is replicated using splenic cDC2 from IRF4-inducible mice, similar Th17 polarization is observed. The inhibited IRF4 expression reduced IL-5 level (Fig. 2.4 d) but increased IL-17a level, suggesting that inhibited IRF4 in DCs promotes Th17 response (Fig. 2.4 e).

To support our founding, we devised another IRF4 deficient mouse model: *IRF4*$^{\Delta CD11c}$ mice model. Since IRF4 is ablated in $CD11c^+$ cells, *IRF4*$^{\Delta CD11c}$ mice's cDC2s ($CD11c^+CD11b^+CD8^-$) also do not express IRF4 (Fig. 2.4f). With previously mentioned procedures, splenic cDC2 from *IRF4*$^{\Delta CD11c}$ mice are co-cultured with OT2 $CD4^+$ T cells to measure the expression of IL-4 and IL-17a. When compared to the control fl/fl group, the IRF4 KO group inhibited secretion of IL-4 (Fig. 2.4g) but encouraged secretion of IL-17a (Fig. 2.4h). Likewise, the co-cultured T cells from *IRF4*$^{\Delta CD11c}$ group displayed inhibited expression GATA-3 compared to that of the fl/fl group (Fig. 2.4i). Yet, the IRF4 KO group also showed a greater induction of RORγt than that of the control (Fig. 2.4i). Together, these results confirm that inhibited expression of IRF4 is sufficient and essential for DCs to induce Th17 differentiation.

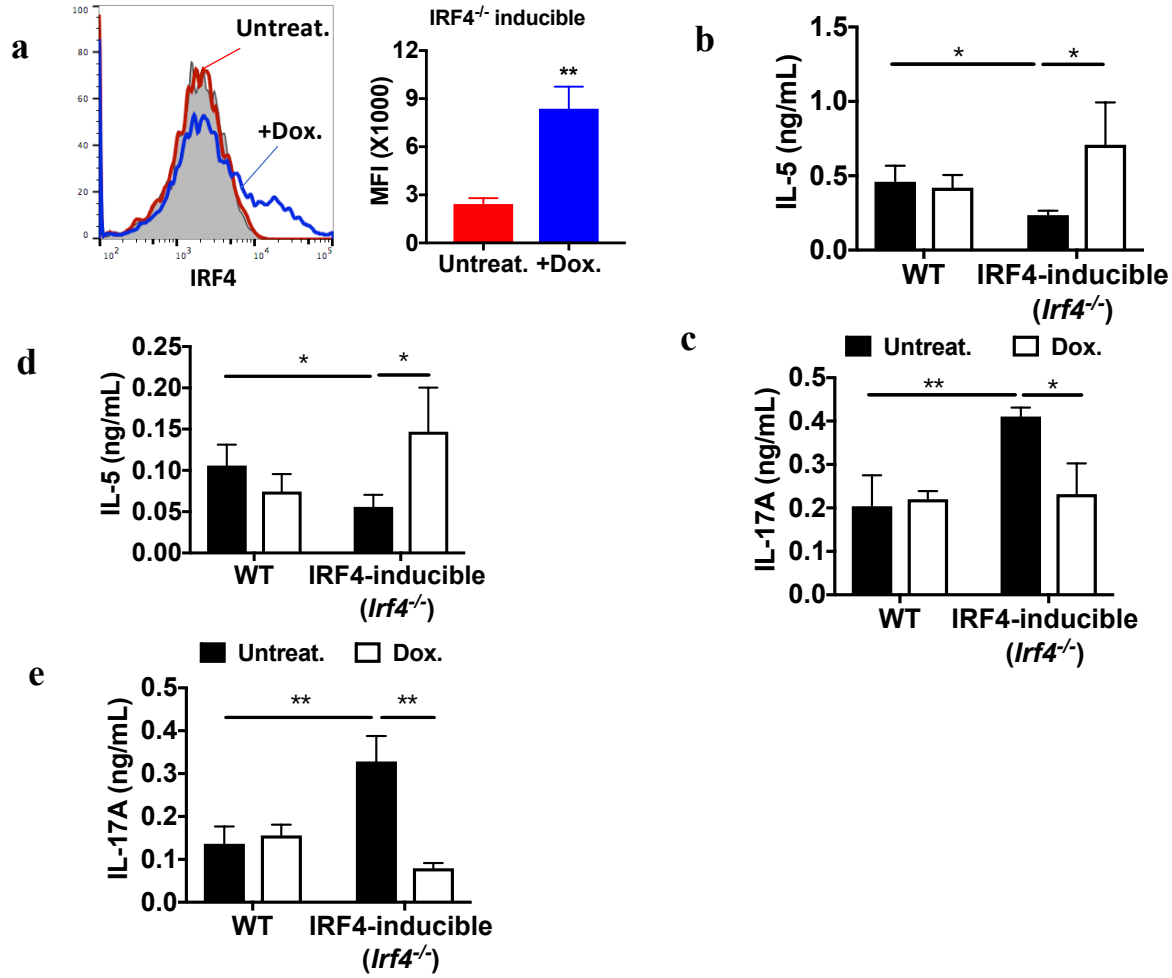



**Figure 2.4. The inhibition of IRF4 expression in DCs is essential in Th17 polarization**

**(a)** the expression of IRF4 for isolated BMDCs from WT and *Irf4*-inducible mice. These BMDCs are either treated with or without doxycycline (Dox). **(b, c)** Cytokine level (IL-5 and IL-17a) of splenic OT2 $CD4^+$ T cells which has been co-cultured with BMDCS from WT and *Irf4*-inducible mice. These DCs are either treated with or without Dox and loaded with OVA. The OT2 $CD4^+$ T cells have been stimulated with anti-CD3 and anti-CD28 antibodies. **(d, e)** Cytokine level (IL-5 and IL-17a) of splenic OT2 $CD4^+$ T cells which has been co-cultured with splenic cDC2 from WT and *Irf4*-inducible mice. These DCs are either treated with or without Dox and loaded with OVA. The OT2 $CD4^+$ T cells have been stimulated with anti-CD3 and anti-CD28 antibodies. Data are mean ± s.e.m, n=3 in each group; * $p<0.05$, ***$p<0.001$.

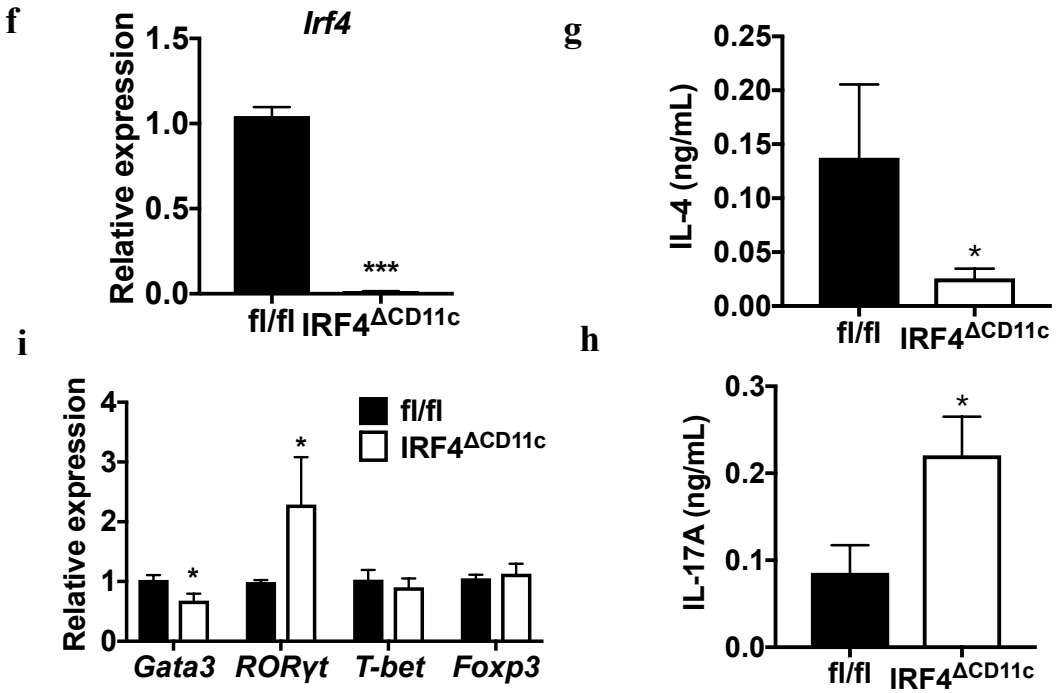



**Figure 2.4. The inhibition of IRF4 expression in DCs is essential in Th17 polarization**

**(d)** the level of (mRNA) IRF4 has been assessed for isolated splenic cDC2 from $IRF4^{fl/fl}$ and $IRF4^{\Delta CD11c}$ mice. **(g, h)** Cytokine level (IL-4 and IL-17a) of splenic OT2 $CD4^{+}$ T cells which has been co-cultured with splenic cDC2 from $IRF4^{fl/fl}$ and $IRF4^{\Delta CD11c}$ mice. These DCs are loaded with OVA. The OT2 CD4+ T cells have been stimulated with anti-CD3 and anti-CD28 antibodies. **(i)** the level of (mRNA) lineage commitment factors (Gata3, RORγt, T-bet, and Foxp3) have been assessed for isolated OT2 CD4+ T cells through qPCR analysis. These T cells are co-cultured with IRF4 KO cDC2. Data are mean ± s.e.m, n=3 in each group; * $p<0.05$, *** $p<0.001$.

### 2.5. DCs' expression of IRF5 is essential for Th17 polarization

IRF5 is another master transcription factor that drives Th17 differentiation for APCs like macrophage [40]. To examine whether IRF5 expression is involved in the cAMP dependent DC reprogramming, we devised an IRF5 deficient animal model: IRF5 $^{-/-}$ mice model. Both splenic cDC2 and BM-DCs from IRF5 KO mice did not express IRF5 under qPCR analysis (Fig. 2.5b and 2.5e). Next, IRF5 KO splenic cDC2s' ability to evoke Th17 response is investigated. With previously depicted methods, cDC2 from IRF5 $^{-/-}$ mice are co-cultured with OT2 $CD4^+$ T cells; some cDC2s groups are also treated with CPT to bias Th17 response. Using ELISA to, the resulting T cell's cytokine profile is collected. CPT treated WT group expressed a higher level of IL17a than the untreated WT group (Fig. 2.5a). However, CPT treated IRF5 KO group did not express a higher level of IL17a than the untreated IRF5 group (Fig. 2.5a). In the absence of IRF5 expression, the IRF5 KO cDC2s failed to prime Th17 response, even upon CPT treatment. IRF8 expression remained relatively unaltered (Fig. 2.5b). Surprisingly, cDC2s with ablated IRF5 gene still inhibited IRF4 and KLF4, after the CPT treatment (Fig. 2.5b). Moreover, the elevated Crem level substantiated that cAMP dependent pathway has been triggered (Fig. 2.5b). Previously, our experiments have shown that DCs' inhibition of IRF4 alone led to Th17 priming. Although IRF4 and KLF4 are downregulated, IRF5 KO cDC2 failed to bias Th17 response. This result suggests that IRF5 is another essential TF that promotes DCs' Th17 polarization.

To verify this intriguing finding, we replicated these experiments using BM-APCs. BM-APCs from IRF 5 KO also failed to prime Th17 response, after CPT treatment (Fig. 2.5c). As splenic cDC2s from IRF5 KO have shown, BM-APCs without IRF5 expression also downregulated IRF4 and KLF4 in a time dependent manner, upon CPT treatment (Fig. 2.5e). Likewise, the Crem level increased which suggested the activation of cAMP dependent pathway (Fig. 2.5e). Again, IRF8 expressions remained unchanged (Fig. 2.5e). Even for BM-DCs, the downregulation of IRF4 is not sufficient to induce Th17 differentiation. In fact, BMDC's expression of IRF5 was also crucial to bias Th17 response.

Together, these results delineate an important aspect about IRF4 and IRF5. Under cAMP dependent DC reprogramming, both BMDCs and splenic cDC2 must downregulate IRF4 expression to provoke Th17 response. Nonetheless, the experiments with IRF5 KO models have shown that both expression of IRF5 and the inhibition of IRF4 must be occurring for DCs to prime Th17 response.

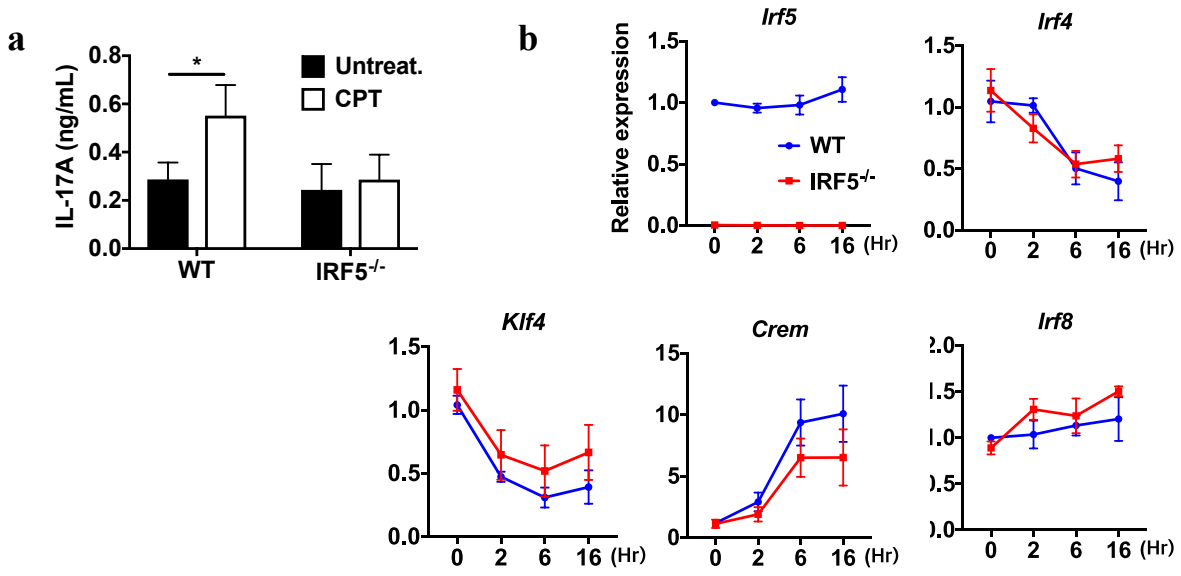



**Figure 2.5. DC's expression of IRF5 is essential for Th17 polarization**

**(a)** Cytokine level (IL-17a) of splenic OT2 CD4+ T cells which has been co-cultured with cDC2s from WT and IRF5$^{-/-}$ mice mice. These DCs are either treated with or without CPT and loaded with OVA. The OT2 CD4+ T cells have been stimulated with anti-CD3 and anti-CD28 antibodies. **(b)** the level of (mRNA) DC TFs (IRF5, IRF4, KLF4 IRF8 and Crem) have been assessed for isolated WT/ IRF5$^{-/-}$ splenic cDC2s through qPCR analysis. These DCs have been either treated with CPT. Two-way ANOVA with Sidak's multiple comparisons test has been applied; Data are mean ± s.e.m, $n$=3 in each group; * $p$<0.05

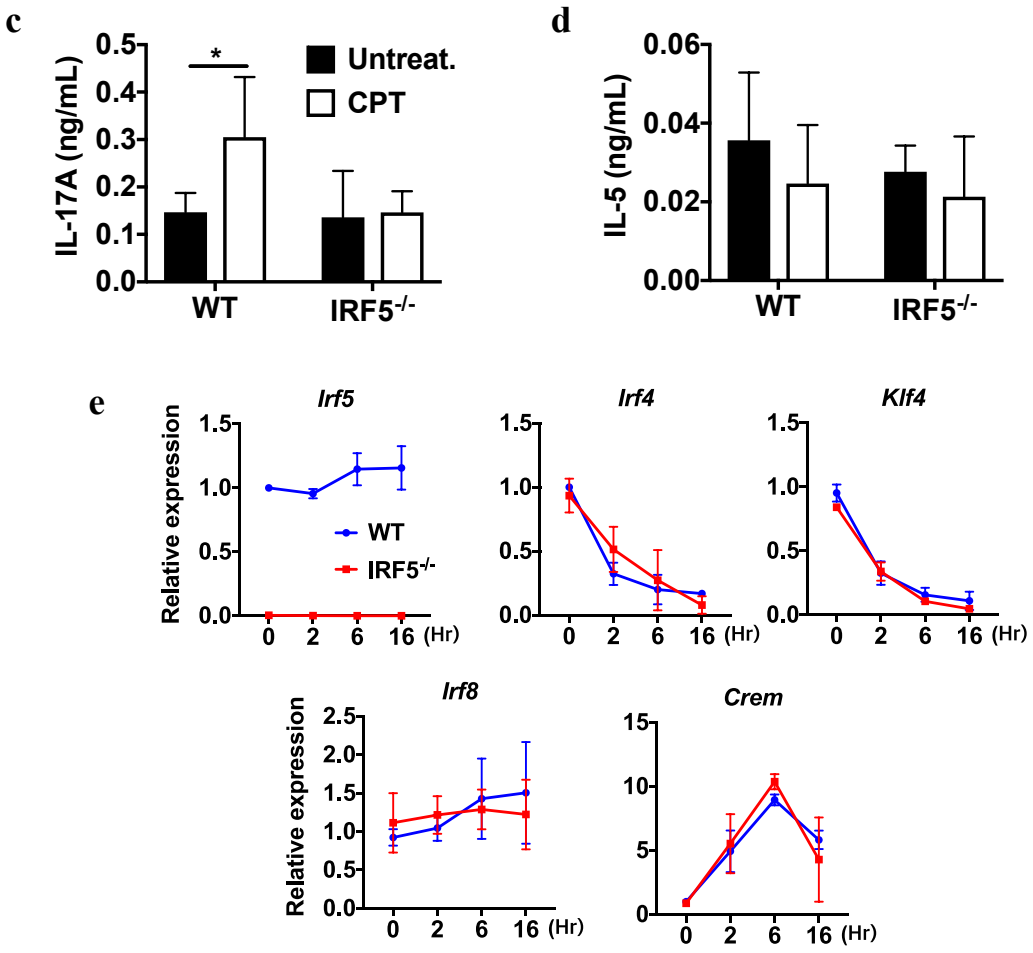



**Figure 2.5. DCs' expression of IRF5 is essential for Th17 polarization**

**(c, d)** Cytokine level (IL-17a and IL-5) of splenic OT2 CD4+ T cells which has been co-cultured with BM-DCs from WT and IRF5$^{-/-}$ mice mice. These DCs are either treated with or without CPT and loaded with OVA. The OT2 CD4+ T cells have been stimulated with anti-CD3 and anti-CD28 antibodies. **(e)** the level of (mRNA) DC TFs (IRF5, IRF4, KLF4 IRF8 and Crem) have been assessed for isolated WT and IRF5$^{-/-}$ BM-DCs through qPCR analysis. These DCs have been either treated with CPT. Two-way ANOVA with Sidak's multiple comparisons test has been applied; Data are mean ± s.e.m, $n$=3 in each group; * $p$<0.05.

This thesis is using materials that is currently being prepared for submission for publication.

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**Chapter 3.**

**RESULT – cAMP signaling induced by β2ARs promotes neutrophilic asthma**

### 3.1 Introduction

Asthmatic patients often take Long-Acting Beta-Agonists (LABA) to breathe easier. Interestingly, LABA is a β2-adrenergic receptor agonist that mediates bronchodilation via cAMP /PKA pathway. Moreover, β-adrenergic receptors (βARs) belong to the GPCR family [41]. Among βARs' three subtypes, β 2-adrenergic receptors (β2ARs) are embedded extensively throughout the lung [42]. Normally, LABA would alleviate the asthma symptom, by promoting bronchodilation of airway smooth muscle and impairing type 2 innate lymphoid cell (ILC2) mediated Th2 response; ILC2 is also known induce eosinophilic asthma[3, 50]. However, a long-term LABA usage has been also linked with the exacerbation of the asthmatic symptoms, resembling the symptoms of neutrophilic asthma [26]. Interestingly, we previously have shown that DCs can also drive either Th2 or Th17 differentiation based on its cAMP level. We postulated that if DCs also express β2-adrenergic receptor, patient's intake of LABA might trigger their lung DCs to elicit Th17 response in the airway. Hence, we hypothesized that LABA might promote neutrophilic asthma by promoting lung DCs to adopt Th17-biasing phenotype.

### 3.2 LABA induces cAMP signaling in WT BMDC leading to Th17 response and transcriptional reprogramming

To address this question, we the first checked whether BM-APCs express β2ARs (Adrb2) as good as airway smooth muscle cells (ASM) express β2ARs; these cells are collected from WT mice [42]. Elevated level of Adrb2 mRNA is shown for both ASM and BM-APC (Fig. 3.2a). In fact, BM-APC produced especially more mRNA for β2AR than other βAR subtypes (Fig. 3.2a). FACS analysis of the β2AR expression also revealed that BM-APCs express β2AR 2-folds higher than ASM (Fig. 3.2b). Next, ASM and BM-APCs are treated with formoterol (LABA) to confirm that activated β2ARs can generate cAMP signaling[26]. Although both ASM and BM-APCs produced a high level of cAMP upon LABA treatment, BM-APCs induced especially heightened cAMP signaling than that of ASM (Fig. 3.2c). These results suggest that BMDCs are fully equipped with β2AR that can trigger cAMP signaling. We then tested whether BMDCs' β2AR/cAMP signaling leads to previously shown Th17-baising TF reprogramming. To investigate this question, DC's mRNA expression of IRF4, KLF4, IRF8 and Crem are measured after LABA treatment. Upon LABA treatment, WT's BM-APCs gradually inhibited the expressions of IRF4 and KLF4 (Fig. 3.2d). IRF8 activity remains unaltered for both groups but Crem expression increased after the LABA treatment (Fig. 3.2d). This result suggests that LABA treatment phenocopied the effect of CPT treatment; BMDC's β2AR /cAMP signaling can also inhibit IRF4 and KLF4 activity.

Subsequently, we examined whether β2ARs' cAMP signaling targets PKA to inhibit IRF4 expression. As formerly shown, the BM-APCs are treated with LABA to initiate cAMP signaling. Some DCs are also treated with PKA or Epac inhibitors to ascertain which downstream effector downregulates IRF4. PKA inhibitor, Rp-cAMP, significantly reverted the effect of LABA, elevating IRF4 level (Fig. 3.2e). However, Epac inhibitor, CE3F4, failed to diminish the effect of LABA (Fig. 3.2e). This result is consistent with our previous findings with CPT. This further support the notion that BMDCs' β2ARs/cAMP/PKA signaling is responsible for its Th17 biasing TF reprogramming. We then seek to confirm that these reprogrammed BMDCs favor Th17 polarization. The BM-APCs are derived from WT mice's bone marrow. Then, BM-APCs are treated with CPT or various β2AR agonist like LABA, epinephrine (Epi) and short-acting β2AR-agonists (SABA). Splenic OT2 $CD4^+$ T cells are cocultured with these DCs to obtain resulting T cells' cytokine profile. When compared to the untreated group, OT-2 $CD4^+$ T cells from all treated groups secreted more IL-17a (Fig. 3.2f). Yet, T cells did not heighten their expression of IL-17a, when they were cocultured with BMDCs treated with LABA and βARs antagonists: propranolol and ICI-118,551 (Fig. 3.2h). Moreover, T cells also upregulated RORγt mRNA expression, upon LABA treatment to BMDCs (Fig. 3.2g). These results support the notion that LABA treatment to DCs induce β2ARs/cAMP signaling, resulting in Th17 response. Additionally, inhibiting PKA prevented LABA mediated IL-17a secretion while inhibiting Epac did not (Fig. 3.2i). Again, the data suggests that BMDC's PKA meditated IRF4 downregulation is involved in promoting Th17 response.

In summary, BM-APCs extensively express β2ARs that trigger cAMP/PKA signaling. When β2ARs agonists like LABA activate DCs' β2ARs, its subsequent cAMP/PKA signaling inhibited IRF4 and KLF4 expression. After this TF reprograming, DCs are shown to favor Th17 response over Th2 response. Hence, LABA is shown to phenocopied the effect of CPT. This BMDC's downregulation of IRF4 is also consistent with our previous results. This result strongly suggests that BMDCs are very susceptible to LABA. LABA can bias Th17 immunity by maturing DCs via cAMP dependent TF reprogramming.

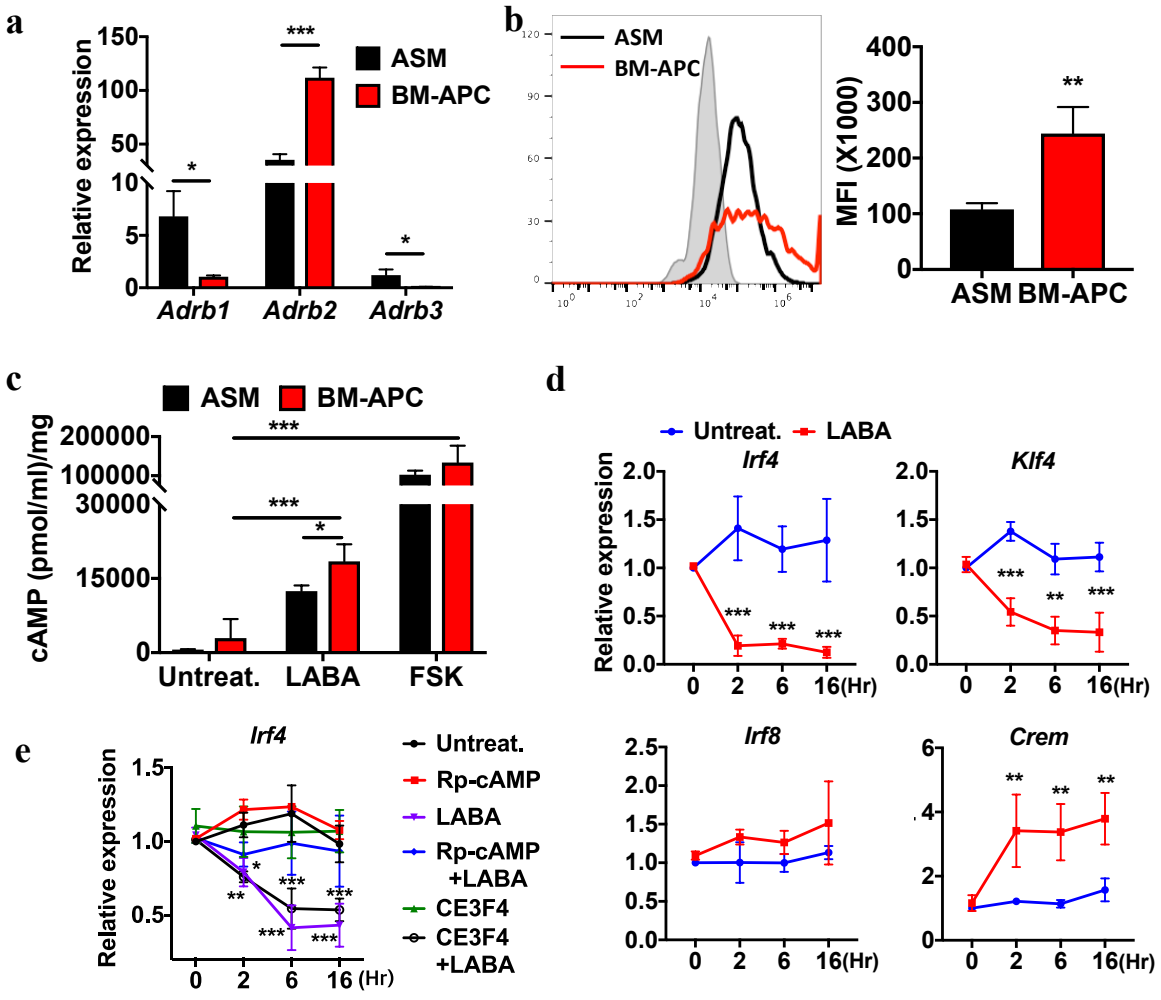



**Figure 3.2. LABA induces cAMP signaling in WT BMDC leading to Th17 response and transcriptional reprogramming**

**(a)**The mRNA expressions of 3 different β adrenergic receptors (Adrb1, Adrb2 and Adrb3) in ASM and BM-DCs are obtained using qPCR analysis. **(b)** β2AR expression of ASM and BM-DCs are obtained using FACS analysis. **(c)** cAMP level of ASM and BM-DCs are obtained upon treatment with long-acting β agonist (LABA) or forskolin (Fsk) using ELISA. **(d)** the level of (mRNA) DC TFs (Irf4, Klf4 Irf8 and Crem) have been assessed for isolated WT BM-DCs through qPCR analysis. These DCs have been either treated with or without LABA. Two-way ANOVA with Sidak's multiple comparisons test has been applied; n=3 in each group; ** p<0.01, ***p<0.001, Effect of LABA; Irf4 (p=0.0046), Klf4 (p<0.001), and Crem (p=0.0013). **(e)** The level of (mRNA) Irf4 has been assessed for isolated WT BMDCs through qPCR analysis. These DCs have been either treated with or without LABA. PKA (Rp-cAMP) or EPac (CE3F4) inhibitors are also applied to some of the groups before the LABA treatment. Two-way ANOVA with Sidak's multiple comparisons test has been applied; *n*=3 in each group, different from untreated; * *p*<0.05, ** *p*<0.01, ***p<0.001.

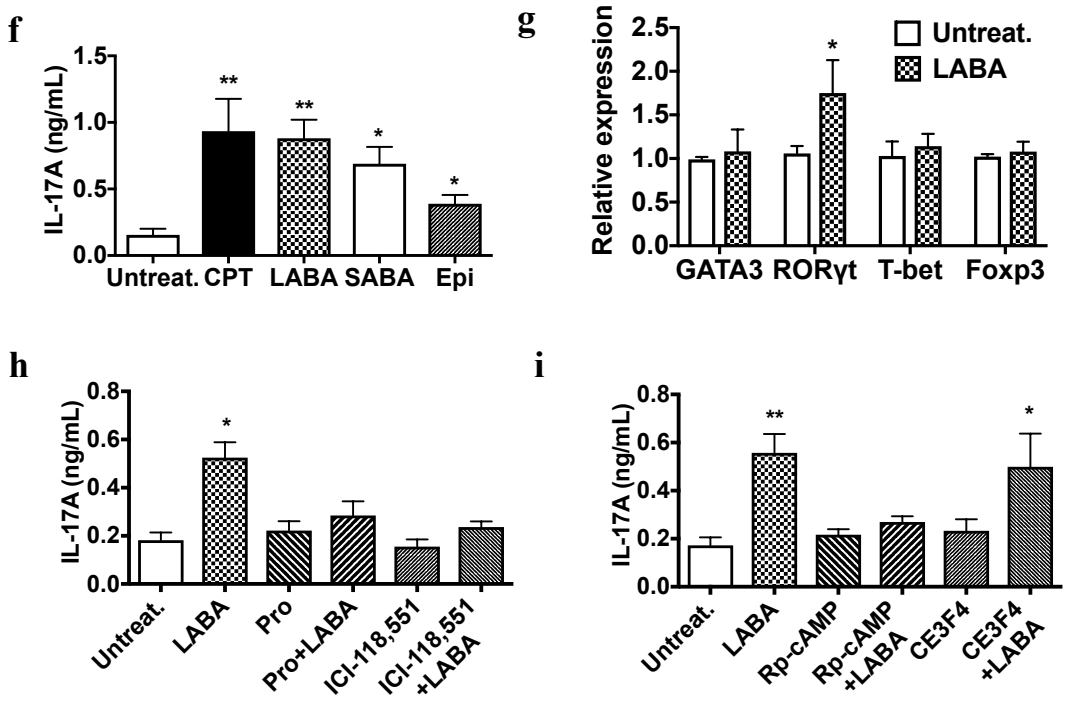



**Figure 3.2. LABA induces cAMP signaling in WT BMDC leading to Th17 response and transcriptional reprogramming**

**(f)** Cytokine level (IL-17a) of splenic OT2 CD4+ T cells which has been co-cultured with BM-DCS from WT mice. These WT DCs are treated with various cAMP elevating molecules: CPT, long-acting β-agonist (LABA), Short acting beta agonists (SABA) and epinephrine (Epi). DCs are loaded with OVA. The OT2 CD4+ T cells have been stimulated with anti-CD3 and anti-CD28 antibodies. **(g)** the level of (mRNA) lineage commitment factors (Gata3, RORγt, T-bet, and Foxp3) have been assessed for isolated OT2 CD4+ T cells through qPCR analysis. These T cells are co-cultured with WT BM-DCs that have been either treated with or without LABA. **(h)** Cytokine level (IL-17a) of splenic OT2 CD4+ T cells which has been co-cultured with BM-DCS from WT mice. These WT BMDCs are either treated with or without LABA and loaded with OVA. Some of these BMDCs were also treated with β2AR antagonists like propranolol (Pro) or ICI-118,551 an hour before the LABA treatment. The OT2 CD4+ T cells have been stimulated with anti-CD3 and anti-CD28 antibodies. **(i)** Cytokine level (IL-17a) of splenic OT2 CD4+ T cells which has been co-cultured with BM-DCS from WT mice. These WT DCs are either treated with or without LABA and loaded with OVA. Some of these DCs were also treated with PKA inhibitor or Epac inhibitor 6 hours before the LABA treatment. The OT2 CD4+ T cells have been stimulated with anti-CD3 and anti-CD28 antibodies. Two-way ANOVA with Sidak's multiple comparisons test has been applied; data are me an ± s.e.m, $n$=3 in each group; * $p$<0.05, ** $p$<0.01, *** $p$<0.001.

### 3.3 Intranasal LABA delivery promotes Th17-mediated inflammation and remodeling in the airway

So far, we have shown that LABA can instruct DCs to bias Th17 response. From these findings, we postulated that LABA intake might trigger lung DCs to elicit Th17 responses in the airway for asthma patients. We also suspected this DC mediated Th17 response can convert the eosinophilic asthma to neutrophilic asthma if a patient is exposed to LABA for a long duration. For instances, a person with eosinophilic asthma would have a Th2 biasing DC population that contribute to his predominant Th2 immunity in the airway. Yet, the Gnas KO DC were previously shown to convert from Th2-biasing DC to Th17-biasing DC, upon CPT treatment. Likewise, a long-term exposure of LABA might convert a portion of these Th2-biasing DCs to Th17-biasing DCs. Afterwards, a mix of Th2 and Th17 promoting DC population will orchestrate an onset of neutrophilic asthma. To test this hypothesis, we the developed two chronic asthma models: the house dust mite (HDM)-induced chronic asthma model and OVA-induced chronic asthma model. In these models, HDM or OVA/ alum are used to initially elicit Th2 response in the mice's lung; mice eventually develop eosinophilic asthma. Then, we planned to switch mice's eosinophilic asthma to neutrophilic asthma using an additional delivery of LABA.

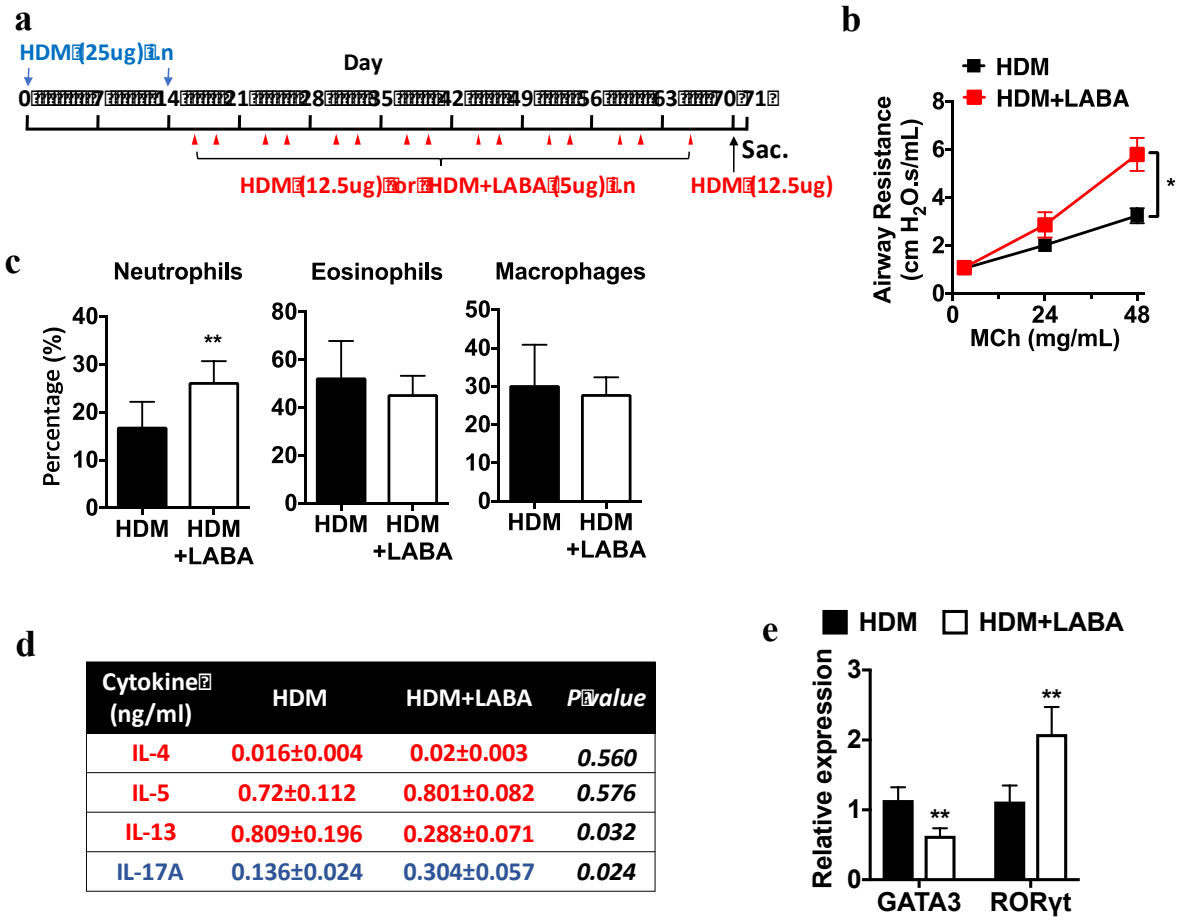
To understand how a long-term LABA administration might affect the progression of asthma, WT mice (C57BL/6) were sensitized by two intranasal (i.n.) deliveries of a house dust mite (HDM). After the sensitization, the mice were re-exposed to either HDM or HDM with LABA twice a week for eight weeks (Fig. 3.3.1a). Methacholine challenge test was performed to determine mice's hyper-responsiveness to methacholine. Mice treated with LABA displayed a greater air way resistance than the control, upon methacholine challenge (Fig. 3.3.1b). This suggests that LABA induced a greater pre-existing airway hyperreactivity than that of the control. LABA treatment appeared to have worsened mice's asthmatic symptom. Moreover, bronchoalveolar lavage revealed that LABA treated mice contain a higher percentage of neutrophils in the lung than the control (Fig. 3.3.1c). To confirm that Th17 cells induced this increased neutrophilic infiltration in the airway, the single-cell suspensions from the mice's lung tissue are used to check the cytokine profile. Administration of LABA led to a lowered production of Th2 signature cytokine like IL13 but also an elevated production of Th17 signature cytokine like IL-17A (Fig. 3.3.1d). The cytokine profile suggests that strong Th17 response is induced in the lung by the LABA treatment. To substantiate this result, T cell lineage markers were evaluated using the lung's single-cell suspensions. When compared to HDM only group, the LABA group downregulated Gata3 and upregulated RORγt (Fig. 3.3.1e). This highlights that lung DCs have been switched from pro-Th2 DCs to pro-Th17 DCs. Although LABA treatment did not upregulated serum IgE and IgG1 levels (Fig. 3.3.1f), histologic analysis of lung tissue

from the LABA group revealed severe features of airway remodeling like airway mucus hypersecretion, fibrosis and mucosal metaplasia (Fig. 3.3.1g and 3.3.1h). We substantiated this heightened airway mucus hypersecretion in LABA group, using qPCR analysis of Muc5ac; heightened Muc5ac expression is known to cause airway mucus hypersecretion [43]. When compared to the HDM only group, the LABA group expressed a higher level of Muc5ac, suggesting that LABA treatment has aggravated mice's asthma, by heightening mucin production (Fig. 3.3.1i). Intranasal delivery of HDM did trigger allergic response in the WT mice. Our data suggest HDM predominantly triggered Th2 immunity in the airway to yield eosinophilic asthma with mild symptoms. Yet, intranasal delivery of additional LABA switched the disease phenotype. Th17 immunity became predominant in the airway. The mice displayed severe asthma symptoms, including excessive airway remodeling. All these characteristics closely resemble the symptoms of neutrophilic asthma. These results strongly indicate that the prolonged exposure to LABA promoted neutrophilic asthma in WT mice.

Our alternative murine asthma model is OVA-induced chronic asthma model. To elicit allergic sensitization, two intranasal deliveries of OVA are administered to C57BL/6 mice with alum as adjuvant. After the sensitization, the mice are re-exposed to either OVA or OVA with LABA twice a week for eight weeks (Fig. 3.3.2a). Methacholine challenge test revealed that LABA treatment led to a greater air way resistance than the control (Fig. 3.3.2 b). These data support the previous findings from the HDM model. Again, LABA administration aggravated asthmatic symptoms. Moreover, LABA mice's BAL fluid contained a higher percentage of neutrophils than the control (Fig. 3.3.2c). The cytokine profile from the lung's single-cell suspensions again highlighted that LABA lowered production of Th2 signature cytokine: IL4, IL5 and IL13. Instead, LABA heightened expression of IL-17a (Fig. 3.3.2d). These LABA treated cells from the OVA model also downregulated Gata3 and upregulated RORγt (Fig. 3.3.2e). This highlights that LABA switched pro-Th2 lung DCs to pro-Th17 lung DCs. LABA administration led to an increased serum IgG1 level which is a feature of Th17 mediated neutrophilic inflammation (Fig. 3.3.2f). Lung histology also showed intensified airway remodelings for the LABA group, including collagen deposition and mucosal metaplasia (Fig. 3.3.2g and 3.3.2h). The mRNA expression of Muc5ac is also elevated for the LABA group which supports the notion that LABA treatment triggered additional neutrophilic inflammation to worsen the symptoms (Fig. 3.3.2i). Overall, OVA-induced chronic asthma model also substantiated our findings from HDM-induced chronic asthma model. Th2-mediated eosinophilic asthma which was elicited by OVA/alum treatment was converted to Th17-mediated neutrophilic asthma when additional LABA treatment was applied.

So far, both the HDM-induced and OVA-induced chronic asthma models strongly suggest that additional LABA administrations promoted Th17 responses in the mice's airway. As a result, the lungs of LABA group exhibited various features of neutrophilic asthma. Yet, we seek to verify this airway-modeling is mediated by Th17 responses. Hence, IL-17a KO mice are used in place of C57BL/6 mice for OVA-induced chronic asthma model. Similar sensitization and challenge protocols have been executed (Fig. 3.3.3a). However, LABA treatment did not alter air way resistance (Fig. 3.3.3b). BAL fluid did not reveal any significant change in neutrophil infiltration between the two groups (Fig. 3.3.3c). LABA treatment did not bias expression of IL-17a (Fig. 3.3.3d). Lung histology showed comparable signs of airway remodeling (Fig. 3.3.3e). Together, without IL-17a, asthma symptoms between the OVA only control group and the LABA treatment group are indistinguishable. This result suggests that Th17 cells' IL-17a is responsible for the symptoms of neutrophilic asthma.

Collectively, two asthma models have shown that additional LABA treatments switch the eosinophilic asthma to neutrophilic asthma in the WT mice. A long-term exposure to LABA promotes Th17 immunity in the mice's airway. Th17 immunity led to symptoms associated with neutrophilic asthma. Our study indicated that IL-17a is responsible for these aggravated symptoms.




| Cytokine (ng/ml) | HDM | HDM+LABA | *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 3.3.1 Intranasal LABA delivery promotes neutrophilic asthma in HDM induced chronic asthma model**

**(a)** the protocol for HDM induced chronic asthma model. **(b)** airway resistance is shown for LABA treated group and HDM only group upon methacholine (MCh) challenge. **(c)** white blood cell composition (percentage-%) in the BAL fluid. **(d)** cytokine profile (IL-4, IL-5, IL-13, 17a) is obtained using ELISA using lung cells from LABA treated and HDM only groups. **(e)** the level of (mRNA) lineage commitment factors (Gata3, RORγt) have been assessed for isolated OT2 CD4+ T cells through qPCR analysis. These T cells are co-cultured with WT BM-DCs that have been either treated with or without LABA. Data are mean ± s.e.m, n=6 mice are used for experiment in each group; * $p<0.05$, ** $p<0.01$.

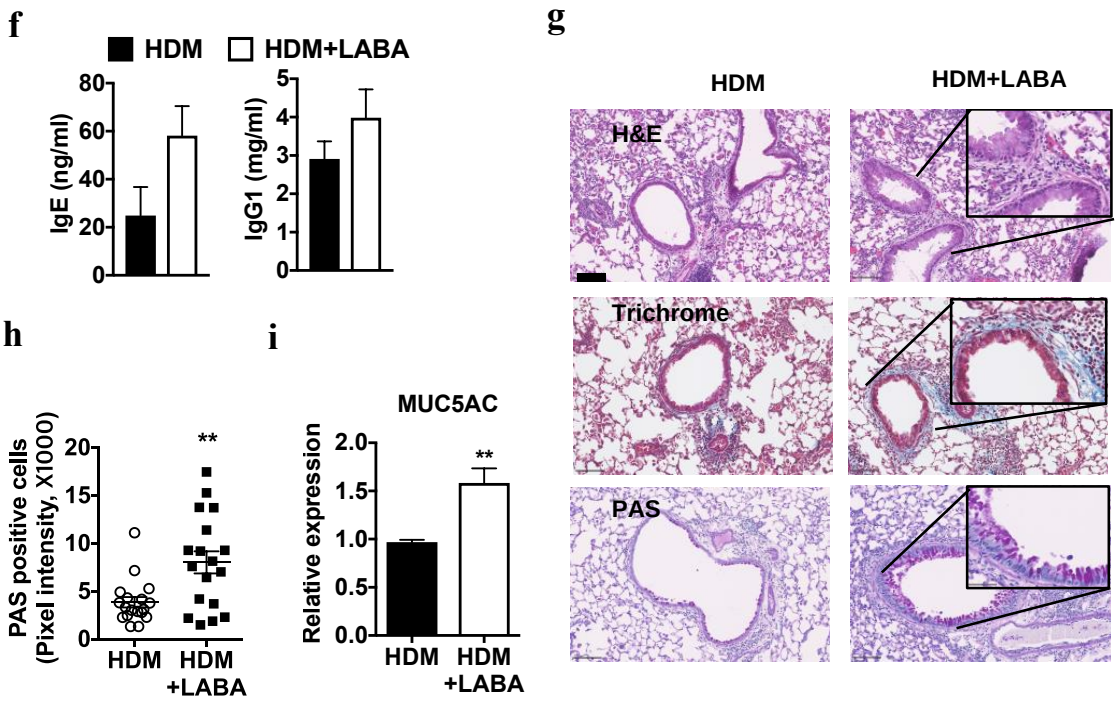
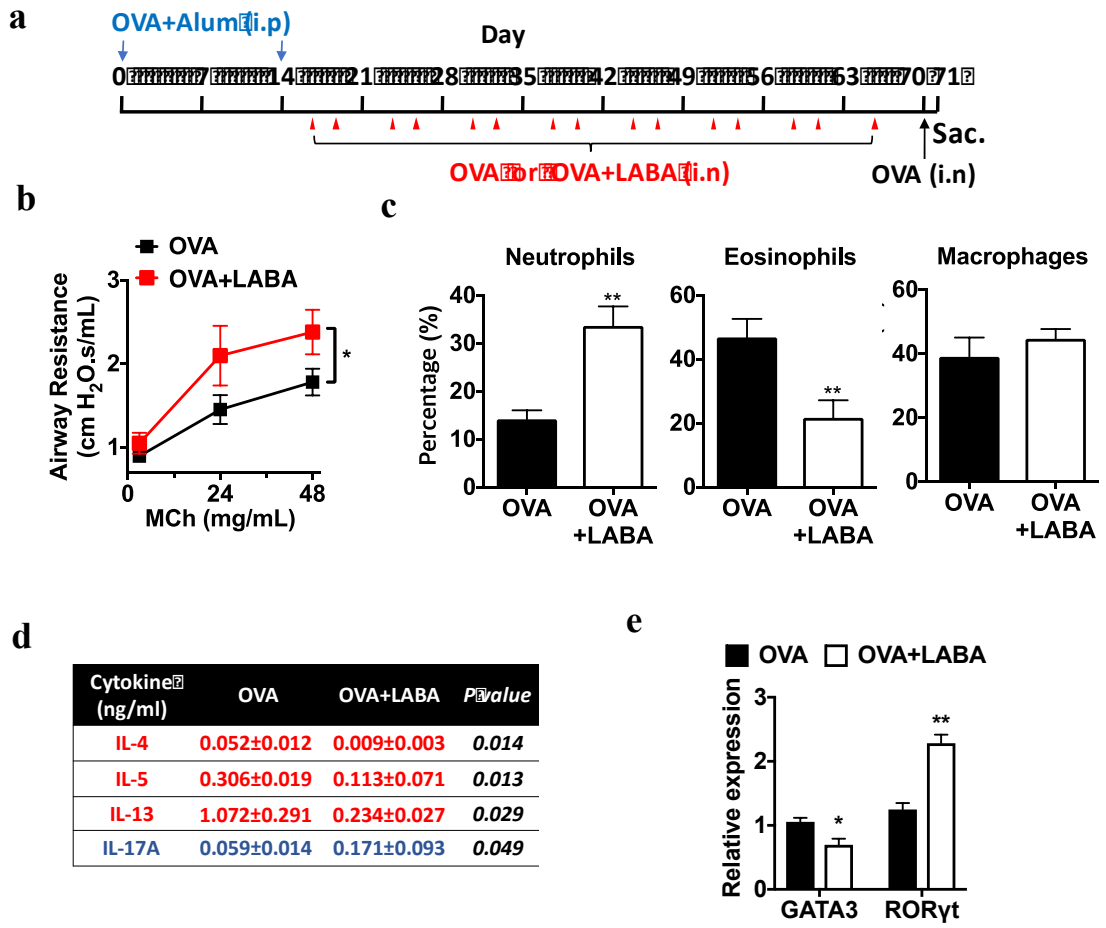



**Figure 3.3.1 Intranasal LABA delivery promotes neutrophilic asthma in HDM induced chronic asthma model**

**(f)** Using ELISA, serum IgE and IgG1 levels are determined from the samples with and without LABA treatment. **(g)** Sections of lung are prepared as histological samples: H&E, blue-Trichrome and red-purple-PAS with magnification ×100, scale bar: 100 μm. **(h)** Total pixel intensity is measured to show PAS positive cells in the PAS histological sample. **(i)** MUC5AC expression (mRNA) is obtained from samples with or without LABA treatment using qPCR analysis. Data are mean ± s.e.m, n=6 mice are used for experiment in each group; * $p<0.05$, ** $p<0.01$.




| Cytokine (ng/ml) | OVA | OVA+LABA | *P value* |
|---|---|---|---|
| IL-4 | 0.052±0.012 | 0.009±0.003 | *0.014* |
| IL-5 | 0.306±0.019 | 0.113±0.071 | *0.013* |
| IL-13 | 1.072±0.291 | 0.234±0.027 | *0.029* |
| IL-17A | 0.059±0.014 | 0.171±0.093 | *0.049* |

**Figure 3.3.2 Intranasal LABA delivery promote neutrophilic asthma in OVA induced chronic asthma model**

**(a)** the protocol for OVA induced chronic asthma model. **(b)** airway resistance is shown for LABA treated group and OVA only group upon methacholine (MCh) challenge. **(c)** white blood cell composition (percentage-%) in the BAL fluid. (d) cytokine profile (IL-4, IL-5, IL-13, 17a) is obtained using ELISA using lung single cell suspension from groups treated with or without LABA. (e) the level of (mRNA) lineage commitment factors (Gata3, RORγt) have been assessed for isolated OT2 CD4+ T cells through qPCR analysis. These T cells are co-cultured with WT BM-DCs that have been either treated with or without LABA Data are mean ± s.e.m, n=6 mice are used for experiment in each group; * $p<0.05$, ** $p<0.01$.

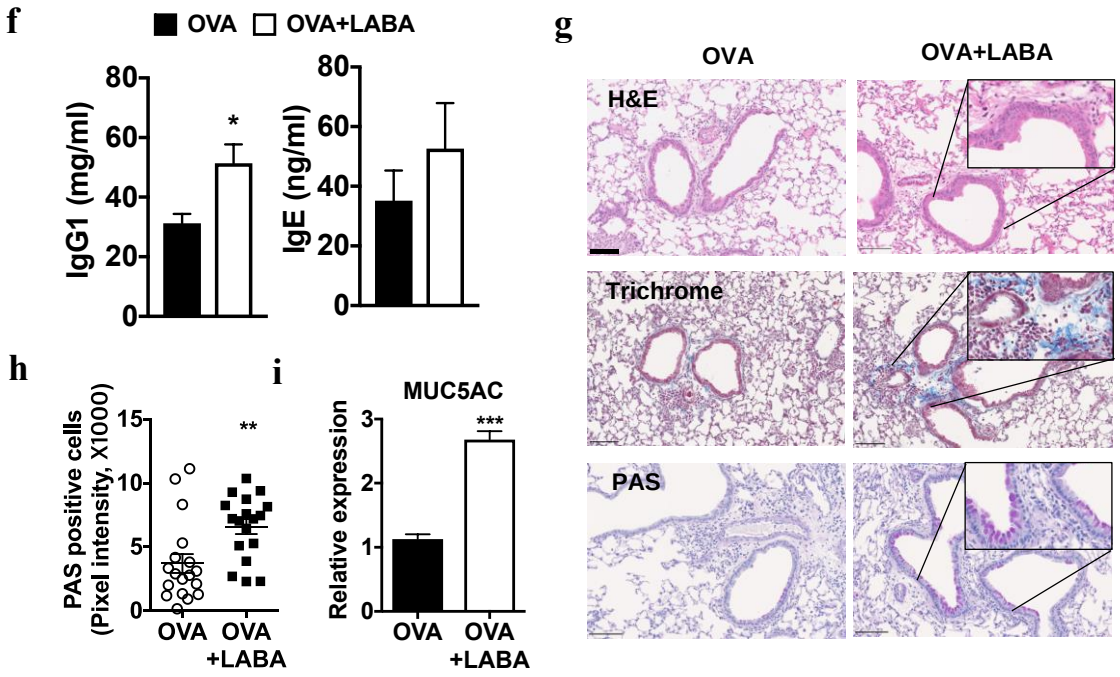
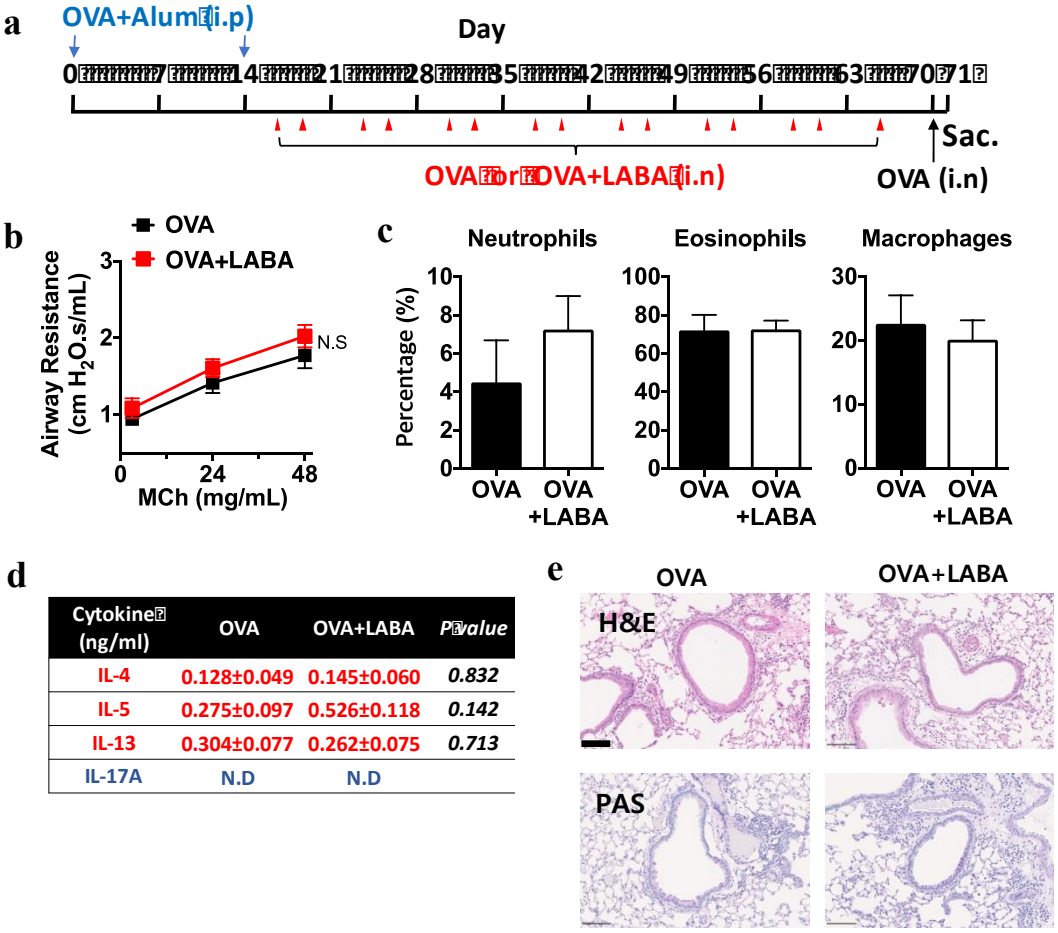



**Figure 3.3.2 Intranasal LABA delivery promote neutrophilic asthma in OVA induced chronic asthma model**

**(f)** Using ELISA, serum IgE and IgG1 levels are determined from the samples with and without LABA treatment. **(g)** Sections of lung are prepared as histological samples: H&E, blue-Trichrome and red-purple-PAS with magnification ×100, scale bar: 100 μm. **(h)** Total pixel intensity is measured to show PAS positive cells in the PAS histological sample. **(i)** MUC5AC expression (mRNA) is obtained from samples with or without LABA treatment using qPCR analysis. Data are mean ± s.e.m, n=6 mice are used for experiment in each group; * $p<0.05$, ** $p<0.01$.




| Cytokine (ng/ml) | OVA | OVA+LABA | *P value* |
|---|---|---|---|
| IL-4 | 0.128±0.049 | 0.145±0.060 | *0.832* |
| IL-5 | 0.275±0.097 | 0.526±0.118 | *0.142* |
| IL-13 | 0.304±0.077 | 0.262±0.075 | *0.713* |
| IL-17A | N.D | N.D | |

**Figure 3.3.3. IL-17a is required to induce LABA mediated neutrophilic inflammation**

**(a)** the protocol for OVA induced chronic asthma model. **(b)** airway resistance is shown for groups with or without LABA treatment upon methacholine (MCh) challenge. **(c)** white blood cell composition (percentage-%) in the BAL fluid. **(d)** cytokine profile (IL-4, IL-5, IL-13, 17a) is obtained using ELISA using lung cells from LABA treated and no treated groups. **(e)** Sections of lung are prepared as histological samples: H&E and red-purple-PAS with magnification ×100, scale bar: 100 µm. Data are mean ± s.e.m, n=6 mice are used for experiment in each group

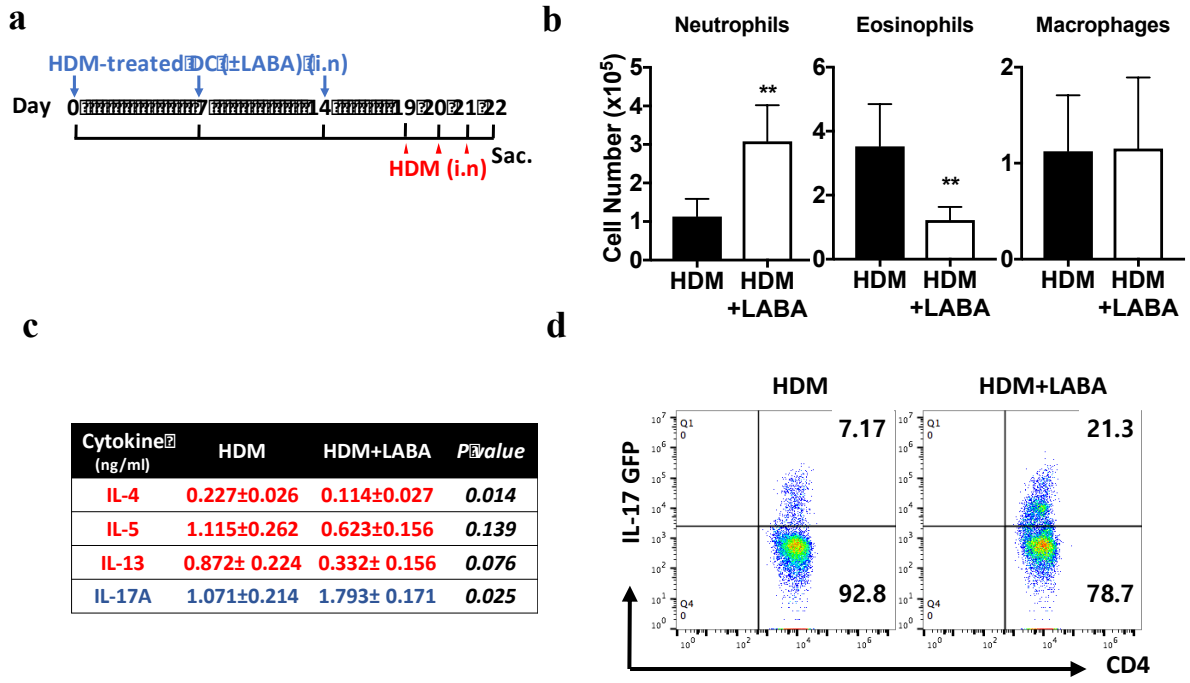
### 3.4. Adoptive transfer of LABA treated BMDC promotes Th17-mediated neutrophilic asthma

Previously, we have administered LABA intranasally to the mice to assess the outcome of treatment. However, such direct delivery of LABA can affect various cells that regulate Th2 response. Namely, type 2 innate lymphoid cell (ILC2) is also known to promote Th2 response via PRR activation [3, 12, 13]. Recent finding suggests that LABA can also impair ILC2's ability to promote Th2 response [3, 50]. Yet, we seek to verify whether Th17-biasing DCs alone can reproduce symptoms associated with neutrophilic asthma via LABA treatment.

To address this question, we devised an adoptive transfer model. BM-APCs from WT mouse is pulsed with HDM. Afterwards, the DCs were either treated with or without LABA. These HDM and LABA treated BM-APCs are intranasally transferred to another WT recipient mice. WT recipient mice were then re-exposed to HDM to elicit allergic response (Fig. 3.4a). Remarkably, the adaptive transfer of these LABA treated BM-APCs alone recaptured the neutrophilic asthmatic features shown with the intranasal delivery of LABA. The LABA-APC treatment groups' BAL fluid contained a higher percentage of neutrophils and lower percentage of eosinophils than the control (Fig. 3.4b). When compared to the HDM only group, the cytokine profile again highlighted that treatment group's DCs lowered the production of Th2 signature cytokine: IL4 and IL13. Also, the adoptive transfer of LABA treated BM-APCs heightened expression of IL-17a (Fig. 3.4c). Again, heightened neutrophilic recruitment is co-observed with a strong Th17 response. These features closely resembled the characteristics of neutrophilic asthma in HDM and OVA-inducible chronic asthma models. Hence, the adoptive transfer of LABA treated BMDC successfully phenocopied the effect of intranasal delivery of LABA.

To support these findings, another adoptive transfer of HDM and LABA treated WT BM-APCs was executed onto IL-17-eGFP mice. Although the recipient mice are now IL-17-eGFP mice, other protocol remained unaltered. Upon the adoptive transfer, the lung cells from the LABA group displayed a greater GFP activity than the control (Fig. 3.4d). This suggests that LABA treated and HDM pulsed BMDCs are fully capable of promoting a strong Th17 response in the lung.

Collectively, our data have shown that BMDCs that are treated with HDM and LABA can be biased to promote Th17 response in the lung. Moreover, the adoptive transfer of these Th17-biasing DCs is sufficient to phenocopy the features shown with the intranasal delivery of LABA. Thus, DCs modified by LABA appear to play a central role in the onset of neutrophilic asthma.




| Cytokine (ng/ml) | HDM | HDM+LABA | *P value* |
|---|---|---|---|
| IL-4 | 0.227±0.026 | 0.114±0.027 | *0.014* |
| IL-5 | 1.115±0.262 | 0.623±0.156 | *0.139* |
| IL-13 | 0.872± 0.224 | 0.332± 0.156 | *0.076* |
| IL-17A | 1.071±0.214 | 1.793± 0.171 | *0.025* |

**Figure 3.4 Adoptive transfer of LABA treated BMDC promotes Th17-mediated neutrophilic asthma**

**(a)** the protocol for adoptive transfer of HDM loaded and LABA treated BMDC. **(b)** white blood cell composition (percentage-%) in the BAL fluid. **(c)** cytokine profile (IL-4, IL-5, IL-13, 17a) is obtained using ELISA using lung single cells suspensions from LABA treated and LABA untreated groups. **(d)** HDM loaded and LABA treated BMDC are transferred onto into IL-17A eGFP mice. After the scarification, lung cells were stimulated with PMA and ionomycin. Then their fluorescence intensity was analyzed under FACS. Data are mean ± s.e.m, $n$=5 recipient mice in each group; ** $p$<0.01.

### 3.5 LABA promotes Th17 response and reprograms transcription factors in WT lung DCs

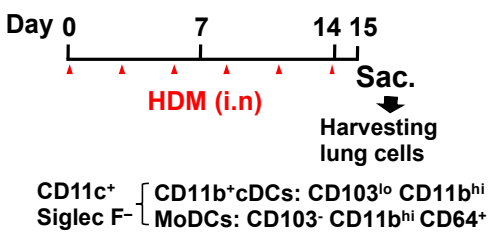
Lung is populated with various DCs subtypes: cDCs, pDCs and MoDCs [44]. Moreover, lungs' cDC2s ($CD11c^{+}$ $SiglecF^{-}$ $CD103^{lo}$ $CD11b^{hi}$ $CD64^{-}$) and MoDCs ($CD11c^{+}$ $SiglecF^{-}$ $CD103^{-}$ $CD11b^{hi}$ $CD64^{+}$) are known to evoke Th2 response during the airway inflammation[45, 46, 47]. Previously, we used BM-APCs to induce Th17 mediated neutrophilic asthma. Despite our success, BMDCs are not resident DCs in the lung. We seek to test whether resident lung DCs can also induce Th17 differentiation via β2ARs dependent pathway.

To address this question, we generated a HDM mediated airway inflammation using WT mice (Fig. 3.4a). After two weeks of HDM challenge, cDC2s and MoDCs from the mice's lung were obtained using FACS sorting. These two DC populations are then treated with LABA and co-cultured with OT-2 $CD4^{+}$ T cells. Remarkably, the cytokine profile of the resulting T cells highlighted that both DC populations can bias Th17 response, upon LABA challenge (Fig. 3.4b). When compared to the untreated control group, LABA treated group lowered production of IL-4 but also heightened expression of IL-17a (Fig. 3.4b). Moreover, both DC subtypes downregulated IRF4 and KLF4, upon LABA treatment (Fig. 3.4c). Moreover, Crem activity heightened, upon LABA treatment. This data suggests that LABA treatment activates β2AR/ cAMP signaling pathway for both lung cDC2 and MoDCs. Subsequently, lung cDC2 and MoDCs also downregulate IRF4 and KLF4 to promote Th17 response.

To verify this finding, we repeated this experiment using OVA/alum immunized mice (Fig. 3.4d). Again, the cytokine profile of the co-cultured T cells verifies that cDC2s and MoDCs can bias Th17 response, upon LABA challenge (Fig. 3.4e). Moreover, cDC2 treated with LABA downregulated IRF4 and KLF4 while they upregulated Crem (Fig. 3.4f). This result is consistent with our previous findings. Lung DCs also appear to adopt cAMP dependent maturation to promote Th17 response.

Together, the data suggest that LABA treatments to both lung resident cDC2s and MoDCs elicit cAMP signaling to inhibit IRF4 expression. This inhibitory TF reprograming allows DCs to promote Th17 response. Hence, these findings strongly support the notion that lung resident DCs should be capable of eliciting neutrophilic asthma, upon a long-term exposure to LABA.

**a**




**b**

| Cytokine (ng/ml) | DC subset | Untreat. | LABA |
|---|---|---|---|
| IL-4 | CD11b+ | 0.179 | 0.075 |
| | MoDCs | 0.112 | 0.052 |
| IL-17A | CD11b+ | 0.052 | 0.092 |
| | MoDCs | 0.044 | 0.125 |

**c**

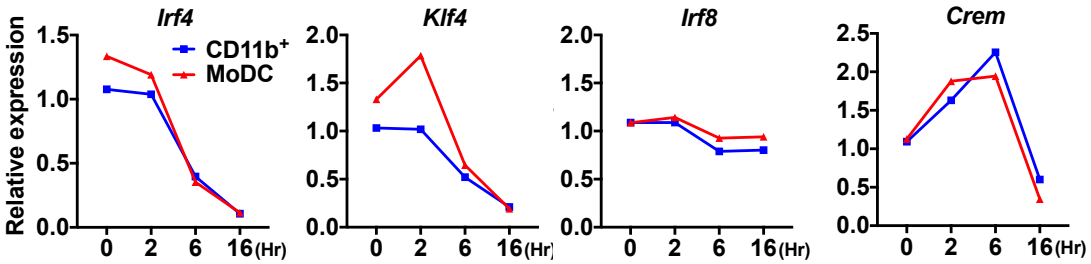
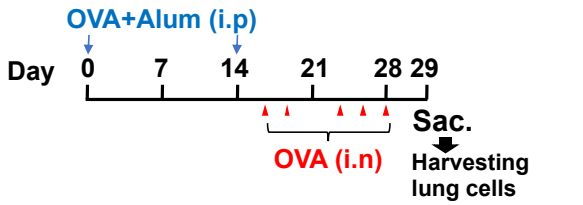



**Figure 3.5 LABA promotes Th17 response and reprograms transcription factors in WT lung DCs**

**(a)** the protocol for HDM mediated airway inflammation **(b)** cytokine profile (IL-4, 17a) of splenic OT2 $CD4^+$ T cells is obtained using ELISA. MoDCs and lung cDCs that have been isolated by FACS are either treated with or without LABA. These DCs were then co-cultured with $CD4^+$ T cells. The OT2 $CD4^+$ T cells have been stimulated with anti-CD3 and anti-CD28 antibodies. **(c)** the level of (mRNA) DC TFs (IRF4, KLF4 IRF8 and Crem) have been assessed for these resident lung-DCs through qPCR analysis. These DCs have been treated LABA.

**d**




CD11c$^{+}$ Siglec F$^{-}$ { CD11b$^{+}$cDCs: CD103$^{lo}$ CD11b$^{hi}$ ; MoDCs: CD103$^{-}$ CD11b$^{hi}$ CD64$^{+}$

**e**

| Cytokine (ng/ml) | DC subset | Untreat. | LABA |
|---|---|---|---|
| IL-4 | CD11b$^{+}$ | 0.773 | 0.315 |
| | MoDCs | 0.596 | 0.358 |
| IL-17A | CD11b$^{+}$ | 0.737 | 1.103 |
| | MoDCs | 0.108 | 0.212 |

**f**

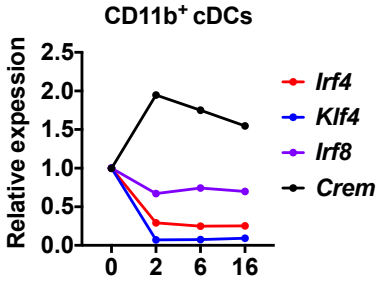



**Figure 3.5 LABA treatments to WT lung DCs promote Th17 response**

**(d)** the protocol for OVA mediated airway inflammation **(e)** cytokine profile (IL-4, 17a) of splenic OT2 CD4$^{+}$ T cells is obtained using ELISA. MoDCs and lung cDCs that have been isolated by FACS are either treated with or without LABA. These DCs were then co-cultured with CD4$^{+}$ T cells. The OT2 CD4$^{+}$ T cells have been stimulated with anti-CD3 and anti-CD28 antibodies. **(f)** the level of (mRNA) IRF4, KLF4 IRF8 and Crem has been assessed for these resident lung-DCs through qPCR analysis. These DCs have been treated LABA.

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**Chapter 4:**

**DISCUSSION AND FUTURE DIRECTION**

**4.1 discussion**

DCs are important sentinels of vertebrates' immune system that provide a link between innate and adaptive immunity [1, 2, 3]. Many recent findings have shown how DCs use its PRRs to induce and control adaptive immunity [2, 10, 11]. PRR signaling within DCs leads to superior antigen processing and presentation as well as the generation of co-stimulatory molecules, inflammatory cytokines and chemokines. After its activation, DCs can coordinate specific T cell responses [48, 49]. Although our understanding of PRRs' roles in shaping adaptive immunity is expanding, the realm of DC-activation by non-PRR signaling has been largely unexplored.

Yet, we aimed here to contribute to the understanding of a novel cAMP-dependent and PRR-independent DC maturation. Throughout the experiment, we found that DC maturation can be initiated by triggering GPCRs coupled to Gs alpha subunit like β2ARs. GPCRs or its Gs alpha subunits were stimulated by various molecules: forskolin, cholera toxin, prostaglandin E2, LABA and SABA; other downstream effectors within GPCR pathway like adenylyl cyclase and Gi alpha subunit were also targeted to increase cAMP level. Subsequently, these treatments initiated DCs' cAMP signaling pathway[42]. Among cAMP's various downstream effector proteins, we have shown that PKA is responsible for downregulating DC's IRF4.

Our genetic models, IRF4 inducible mice and IRF4$^{\Delta CD11c}$ mice, showed that the inhibition of IRF4 expression alone can promote to Th17 polarization. Hence, inhibition of IRF4 is shown to be essential in promoting Th17 response. The results from these models displayed a striking resemblance to the results from DCs with artificially elevated cAMP levels. Collectively, this suggests that cAMP signaling eventually inhibits DCs' IRF4 expression via PKA. In fact, this TF reprogramming also appears to be present in Dectin-1 dependent Th17 differentiation. We elicited this PRR dependent pathway using curdlan and confirmed that neither cAMP nor PKA activity is heightened. Nonetheless, DCs also downregulated IRF4 and KLF4 to drive Th17 response. Likewise, IRF4 downregulation is appeared to be utilized by both PRR dependent and independent DC maturation. Perhaps, both maturations share another enzyme that allows downregulation of IRF4. Another murine model, IRF 5 KO mice, highlighted that DC's expression of IRF5 is also essential to promote Th17 differentiation. When we manually suppressed the IRF 5 deficient DCs' expression of IRF4 and KLF4, these DCs were still not able to induce Th17 response. In short, both the expression of IRF5 and inhibition of IRF4 seem to be required to generate

pro-Th17 DCs. Various DC populations such as BM-APC, splenic cDC2, lung cDC2 and lung MoDC were controlled by this cAMP signaling pathway to promote Th17 differentiation

$CD4^+$ T cells which were co-cultured with these pro-Th17 DCs have presented various features of Th17 cells. These T cells secreted lower levels Th2 signature cytokines and a higher level of Th17 signature cytokine [27] [28]. Moreover, $CD4^+$ T cells displayed a unique set of lineage-determining TFs. Many co-cultured T cells upregulated RORγt and downregulated GATA-3; Th2 and Th17 cells express GATA-3 and RORγt, respectively [3, 12]. Indeed, these results confirm that our pro-Th17 DCs triggered Th17 response via cAMP dependent pathway. In fact, this cAMP/PKA dependent DC reprogramming is so robust that it switched *Gnas*$^{\Delta CD11c}$ mice's Th2-biasing DCs to Th17-biasing DCs.

We do not think DCs' PRRs are activated to bias the T cell response in these experiments. Throughout the experiment, we used antibiotics while culturing these DCs and OT2 splenic $CD4^+$ T cells. Although fl/fl mice were co-housed the KO mice, fl/fl mice did not displayed either Th2 or Th17 responses without the administration of cAMP elevating molecules. WT mice's DC without any treatment also did not bias either Th2 or Th17 response. DCs provoked either Th2 or Th17 response due to genetic alteration and our chemical treatments. Collectively, it is highly unlikely for microbial products to have influenced our DCs to impact T cell response.

Our studies further emphasize the cAMP dependent DC maturation is applicable *in vivo* using two asthma models: HDM-induced chronic asthma model and OVA-induced chronic asthma model. In these experiments, the mice's lung displayed aggravated symptoms of asthma (severe inflammation, collagen deposition and mucosal metaplasia), upon additional and long-term LABA administration. Their BAL fluids showed an increased neutrophil infiltration into the lung. Moreover, the T cells from the lung have lowered production of Th2 signature cytokines and heightened expression of IL-17a. Our analysis suggests that LABA treatment can trigger lung DCs to favor Th17 response; LABA would trigger Th17 response by activating DC's β2ARs to attenuate cAMP/PKA signaling (Fig. 4). LABAs are frequently used to treat asthma because LABAs promote bronchodilation via GPCR signaling pathway [26]. However, a long term and excessive use of LABA has been linked with exacerbation of the asthmatic symptoms [26]. Unfortunately, neutrophilic asthma often displays severe symptoms and resistance to the current treatment [24]. Notably, asthma patients displaying severe symptoms also have displayed features of neutrophilic asthma; their BAL fluid showed an increased neutrophils percentage and an elevated secretion of Th17 signature cytokines such as IL-17A, IL-17F, and IL-22 [24, 25]. These reports from human patients demonstrate striking resemblances to our results. These observations strongly support the notion

that the LABA administration might also promote neutrophilic asthma in humans. Hence, it should be of benefit to integrate this principle into therapeutic regimen for asthma, bearing in mind that current LABA treatment might entails an unforeseen side effect of inducing neutrophilic asthma.

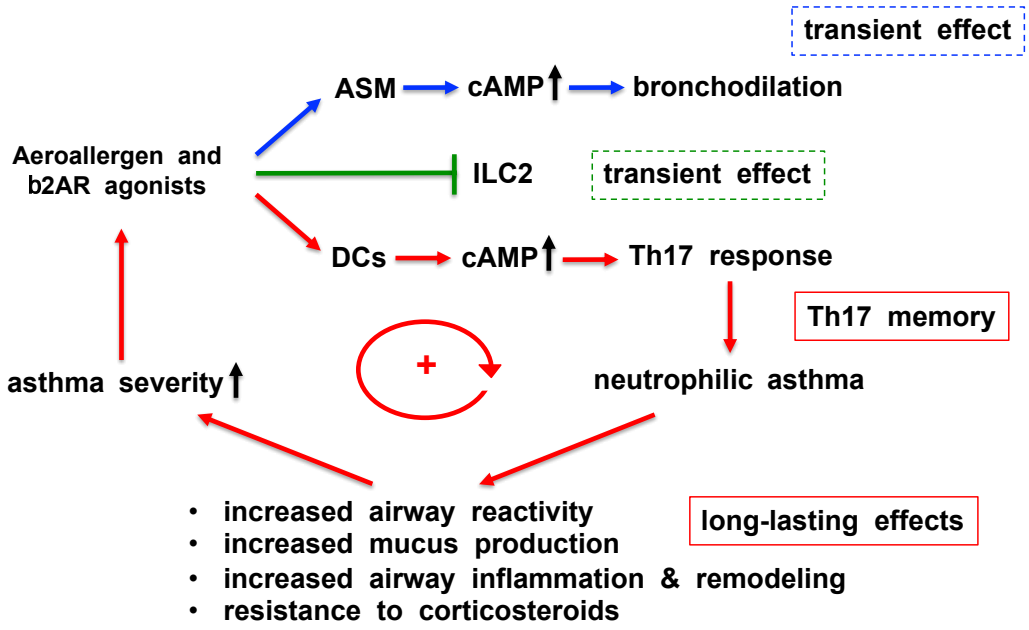


**Figure 4 Schematic of novel DC activation pathway and its potential implication on asthma**

LABA is known to cause bronchodilation by activating β2AR of airway smooth muscle cells. LABA also inhibit the ILC2 from promoting Th2 response; this is thought to moderate the symptoms of eosinophilic asthma. However, our experiments have shown that LABA can also influence DCs to induce its cAMP signaling pathway. DCs prime Th17 response through cAMP/PKA pathway. Consequently, prolonged and persistent activation of this pathway by LABA treatment leads to onset of neutrophilic asthma.

### 4.2 Future direction

Since we had used murine models in our studies, we could not accurately determine the role of LABA in human DCs. Further experiment using human DCs would certainly enhance our understanding for cAMP dependent DC's maturation. Both Dectin-1 dependent and cAMP dependent DC's maturation downregulated IRF4. However, we could not identify a common regulator for both pathways to inhibit IRF4. Identifying this important protein will greatly enhance our understanding of molecular mechanism behind Th17-biasing DC's maturation. Our study also fails to address stimuli produced by DCs such as cytokines that are used to prime Th17 response. Identifying these DC stimuli is another important topic that needs to be addressed. Additional aim for the future studies would be whether DC's cAMP meditated T cell response can also impact T-cell memory. Finally, considering the discoveries described here a greater exploration of GPCR or other non-PRR mediated DCs' maturation seems warranted.

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## MATERIALS AND METHOD

### Animals

C57BL/6J, CD11c-Cre transgenic, OT-II, IL17A-eGFP and *Irf4*$^{fl/fl}$ are all obtained from the Jackson Laboratory. loxP-flanked *Gnas* mice are crossed with CD11c-Cre mice to generate *Gnas*$^{\Delta CD11c}$ mice in our lab. *Irf4*$^{fl/fl}$ mice are crossed to CD11c-Cre mice to produce IRF4 KO mice. GFP expression in these mice are used to verify the deletion of IRF4 in the DCs (96% of the splenic cDC2 showed to be GFP positive). For control, fl/fl littermates have been used. Dr. R. Locksley from University of California San Francisco and Dr. M. Kronenberg from La Jolla Institute For Allergy and Immunology donated IL4-eGFP reporter (4Get) mice. We crossed IL4-eGFP reporter mice with OT-2 mice to generate IL4-eGFP /OT2 and IL17a-eGFP/OT2 mice. Dr. R. Sciammas from University of California, Davis donated IRF inducible mice. Dr. I. R. Rifkin from Boston University donated IRF 5 inducible mice. Dr. Y. Iwakura from University of Tokyo donated IL17a KO mice. All mice are harbored in a specific pathogen free facility.

### Cells

BM cells are harvested from mice's bone marrow. Then, BM cells were cultured with GM-CSF (10ng/ml) for 7 days. After a week of culture, the floating cells are harvest. Through FACS sorting, we isolated $CD11c^{+}CD135^{+}$ cells (BMDCs). Splenic OT2-T cells are obtained using EasySep™ Mouse Naïve $CD4^{+}$ T Cell Isolation Kit (StemCell Technologies). Splenic OT2-T cells and BMDCs used for cocultured and for qPCR. Splenic cDC2 cells ($CD11c^{+}CD11b^{+}CD8^{-}$ ) were obtained using FACs sorting. Splenic cDC2 are also used for cocultured and for qPCR. Lungs DCs are obtained from the mice's three lung lobes. We then used FACS sorting to isolate lung cDC2 ($CD11b^{hi}$ $CD64^{-}$ and $CD103^{hi}$ $CD11b^{lo}$ ) and MoDC ($CD103^{-}$ $CD11b^{hi}$ $CD64^{+}$). ASM cells from C57BL/6J mice are obtained from Cell Biologics. ASM cells are also cultured in the provided smooth muscle cell medium.

### Lung DCs isolation

6 C57BL/6J mice are administrated with HDM (25μg*)* to generate a lung airway inflammation. HDM is administered for 3 times/ week for 14 days. 6 C57BL/6J mice are administrated with OVA (200μg) and alum (500μg) for 3 times/ week for 14 days to generate a lung airway inflammation. The day after last OVA or HDM challenge, mice are sacrificed. Initially, heart perfusion is performed using HBSS with 1mM EDTA. 5 lobes of

lungs are then collected and chopped into small pieces. Resulting lung fragments are digested using HBSS with 20μg/ml DNase, 0.5 mg/ml type 1A collagenase and 5% FBS; DNase and collagenase are obtained from Sigma. After 30 minutes of digestion, the cells are pushed through a 70 μm cell strainer. Resulting single cells were used to isolate lung DCs through FACS sorting. Fc receptors are blocked using CD16/CD32 Abs; cells are incubated for 10 minutes using these antibodies. Then cells are stained for appropriate surface markers for 30 minutes.

**Intracellular staining and flow cytometry**

BD Biosciences' C6 Accuri flow cytometer and FlowJo Software are used. Initially, cells are washed using FACS buffer (PBS with 2 % FCS). Then cells are treated with appropriated antibody. Next, the cells are incubated on ice for 30 minutes. After the incubation, cells are washed twice with FACS buffer (PBS with 2 % FCS). Resulting cells are used for FACS analysis. CPT treated cells are used for IRF4 intracellular staining. First, cells are fixed and permeabilized using Cytofix/Cytoperm$^{TM}$ (BD Biosciences). These cells are then stained with IRF4 antibodies and incubated on ice for 30 minutes. After the incubation, cells are washed 3 times with permeabilization buffer. Resulting cells are used for FACS analysis. CD4+ T cells and lung single cells are used to detect eGFP$^{+}$ cells. Initially, cells are stimulated with PMA (50 ng/ml) and ionomycin (1μM) with GolgiStop (BD Pharmingen). After 6 hours of stimulations, the resulting cells are used for FACS analysis.

**Reagents**

We purchased OVA from Worthington Biochemical and from GenScript. Rp-cAMP is obtained from Biolog. Cell labeling antibodies are obtained from eBiosciences and BD Pharmingen.We purchased 8-CPT-cAMP, epinephrine, forskolin, PGE2, doxycycline isoproterenol, PTX, dexamethasone and PMA/ionomycin from Sigma-Aldrich. Curdlan is obtained from Wako Chemicals. HDM is purchased from Greer.We obtained cholera toxin from list biological laboratories. We got anti-mouse CD3/CD28 from BioXcell. Hemifumarate, propranonol, rolipram, hemisulfate, formoterol and CE3F4 are obtained from Tocris.

**ELISA**

Cytokine levels (IL-4, IL-5, IL-13, IL-17a and IFN-γ) in the CD4+ T cells' supernatant obtained using appropriate ELISA kits from eBioscience. We followed manufacturers' instructions to detect the cytokine profile.

**Quantitative PCR**

First, we isolated RNA from the samples using RNA purification Kit from Thermo Fisher Scientific. We followed manufacturer's instructions to harvest our RNA. Using these RNA, we generated cDNA with Superscript III First-Strand system from Invitrogen. We used resulting cDNA and SYBR Green PCR Master Mix from Thermo Fisher Scientific to carry out qPCR. GAPDH is used to normalize the data. Taqman primers are used to detect IRF5 activity. We also followed manufacturer's instructions to carry out qPCR. Our primer sequences are listed as follows:

*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

**Coculture using DCs and OT-2 cells**

Various DCs (splenic cDC2, lung cDC2, lung MODCS, BMDCs) are isolated by FACs sorting. Subsequently, they were treated with appropriate treatments and loaded with OVA (100 $\mu$g/ml). BMDCs and cDC2 from IRF4 KO and IRF4 inducible mice are loaded with MHC class II OVA peptide (1$\mu$g/ml); they are co-cultured with OT2- cells after 2 hours of incubation. cAMP elevating molecules are added with OVA. Curdlan is added with OVA; coculture is performed after 24 hours. IRF4 inducible mice's BMDCs and cDC2 are treated with doxycycline (200 ng/mL) to induce IRF4 expression; after 24 hours the DCs are cocultured with OT2 CD4+ T cells. The DCs are incubated in complete PRMI 1640 of 16 hours with OVA. Afterward, DCs are co-cultured with OT2-CD4+ T cells for 3 days in a serum free medium with additional albumin. BMDCs ($0.5x10^6$ cells) and OT2 T cells ($0.5x10^6$ cells) are cocultured. Splenic cDC2 ($0.3x10^6$ cells) and OT2 T cells ($0.15x10^6$ cells) are cocultured. Lung resident DCs ($0.3x10^6$ cells) and OT2 T cells ($0.15x10^6$ cells) are cocultured. Afterwards, OT2 T cells are isolated. Then, T cells are re-stimulated with anti-CD3/28 antibodies which are bound to plate. After 24 hours of stimulation, supernatants are collected to provide cytokine profile. T cells are also stimulated with PMA/ ionomycin for 3 hours perform qPCR to get T cell lineage markers.

**cAMP assay**

For 30 minutes, ASM cells and BMDCs are co-cultured in RPMI 1640 medium with 10% FCS at 37°C. These cells are then treated with LABA or Fsk; then they were again incubated in previous condition. Then,

medium is aspirated and 7.5 % trichloroacetic acid (50 µl/ million cells) is added. cAMP is then detected and properly normalized; we followed the manufacturer's (Cayman Chemical) instructions.

**Chronic asthma murine models**

Initially, C57BL/6J mice are sensitized with HDM (25µg) on day 0 and day 14 to generate HDM induced asthma. Successively, HDM (12.5µg) or HDM (12.5µg) with LABA (5 µg) are challenged for 2 times/week for 8 weeks. On day 70, HDM (12.5µg) only challenge was administered. IL-17A KO and C57BL/6J mice are used for OVA induced asthma. OVA (50 µg) with Alum (0.5 mg) are used to sensitize the mouse on day 1 and day 14. Successively, with OVA (20 µg) or OVA (20 µg) with LABA (5 µg) are challenged for 2 times/week for 8 weeks. On day 71, methacholine challenge test is performed on these mice to assess their airway hyper-responsiveness. Successively, bronchoalveolar lavage is performed to assess cellular composition in the BAL fluid and IgE/IgG1 level in the serum. Historical analysis is performed using the lung tissues. Cytokine profiling from the lung cells are performed.

**Adoptive transfer**

We utilize the adoptive transfer of BMDCs as the technique is illustrated in Lambrecht B. et al[23]. C57BL/6J mice are used as the donor group. For 24 hours, WT BMDCs are treated with HDM (100µg/ml); they were also treated with or without LABA (5µM). Afterwards, DCs are washed with PBS for two times. The resulting cells are resuspended in PBS ($0.1 \times 10^6$ cells per10 µl PBS) to be used as donors for adoptive transfer. C57BL/6J and IL-17eGFP mice are used as the recipient groups. Previously mentioned DCs are transferred to recipient mice on day 0, day 7 and day 14. Successively, recipient mice are administered with HDM (12.5 µg) on day 19, day 20 and day 21. On day 22, mice are sacrificed to be analyzed.

**Statistical analysis**

GraphPad Prism software is used. For data with 2 groups, student's t-tests are used. For multiple groups ANOVAs are used.

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