

PKC λ /i regulates Th17 differentiation and house dust mite-induced allergic airway inflammation[☆]



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ABSTRACT

Asthma is a chronic airway inflammation in which Th2 and Th17 cells play critical roles in its pathogenesis. We have reported that atypical protein kinase (PKC) λ /i is a new regulator for Th2 differentiation and function. However, the role of PKC λ /i for Th17 cells remains elusive. In this study, we explored the effect of PKC λ /i on Th17 cells in the context of ex vivo cell culture systems and an in vivo murine model of allergic airway inflammation with the use of activated T cell-specific conditional PKC λ /i-deficient mice. Our findings indicate that PKC λ /i regulates Th17 cells. The secretion of Th17 effector cytokines, including IL-17, IL-21 and IL-22, were inhibited from PKC λ /i-deficient T cells under non-skewing or Th17-skewing culture conditions. Moreover, the impaired Th17 differentiation and function by the PKC λ /i-deficiency was associated with the downregulation of Stat3 and Ror γ t, key Th17 transcription factors. We developed a model of Th17 and neutrophil-involved allergic airway inflammation by intratracheal inoculation of house dust mites. PKC λ /i-deficiency significantly inhibited airway inflammations. The infiltrating cells in the lungs and bronchoalveolar lavage fluids were significantly reduced in conditional PKC λ /i-deficient mice. Th17 effector cytokines were reduced in the bronchoalveolar lavage fluids and lungs at protein and mRNA levels. Thus, PKC λ /i emerges as a critical regulator of Th17 differentiation and allergic airway hyperresponsiveness.

1. Introduction

Asthma is a chronic airway inflammatory disease affecting millions of people in the world. CD4⁺ T helper (Th) cells play central roles in its pathogenesis [1, 2]. Naïve CD4⁺ T cells can be differentiated into various subsets, such as Th1, Th2, Th17, and regulatory T (Treg) cells [3–5]. These subsets exert different functions through the secretion of distinct effector cytokine profiles. For example, Th1 cells secrete IFN- γ and IL-12 and are essential for cell-mediated immune responses against intracellular pathogens and autoimmunity. On the other hand, Th2 cells produce IL-4, IL-5 and IL-13, and are important in the control of humoral immunity, anti-worm responses and allergy. The more recently discovered Th17 cells secrete their effector cytokines, such as IL-17, IL-

21 and IL-22, to exert immune responses for intercellular pathogens. Th17 cells are believed to participate in the immunopathogenesis of more wide ranges of diseases, such as autoimmune and allergy [6–9]. Treg cells generally down-regulate immune responses to diminish tissue damage [10, 11]. Most of our understanding for Th differentiation, such as transcription factors and signaling pathways, come from in vitro study [5]. The precise regulation mechanisms of these Th subsets, especially in vivo, remain to be elucidated. Regarding the roles of various Th subsets in the airway inflammation, the enhanced Th2 responses and eosinophil infiltration in the airways are traditionally believed as characteristics of allergic asthma [12–15]. Recently, it is found that other T cell subsets, such as Treg [11, 16] and Th17 cells [17–20] are actively involved in the pathogenesis of airway inflammation,

Abbreviations: BAL, bronchoalveolar lavage; HDM, house dust mite; KO, knock-out; PKC, protein kinase C; Th, T helper; WT, wild-type

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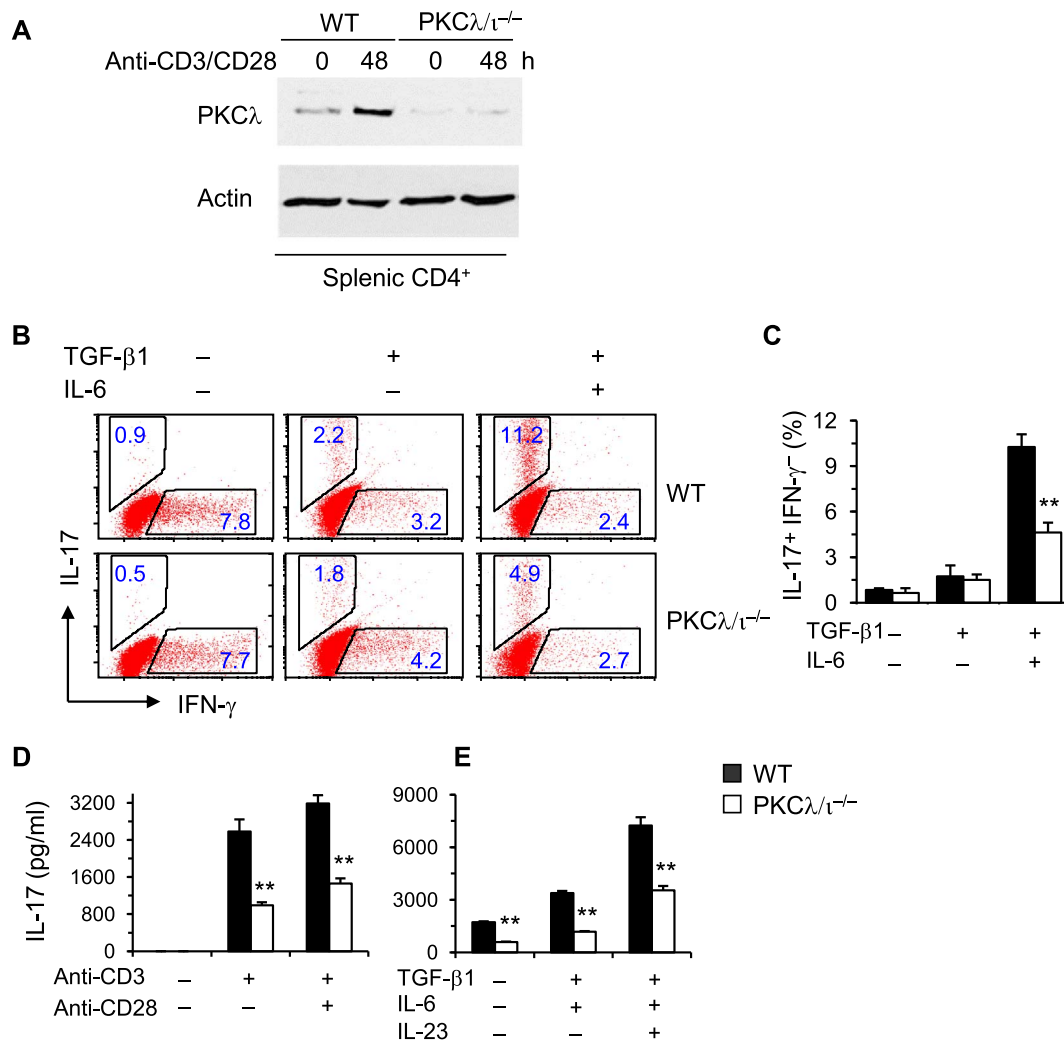


Fig. 1. PKCλ_i-deficiency suppresses Th17 differentiation and cytokine secretion.

A: Immunoblot of PKCλ_i expression in purified splenic CD4⁺ T cells from WT (PKCλ_i^{flx/flx} OX40^{Cre}) and PKCλ_i^{-/-} (PKCλ_i^{flx/flx} OX40^{Cre}) mice. **B–C:** Naïve CD4⁺ T cells were differentiated under Th17 skewing conditions for 3 d and restimulated with PMA plus ionomycin for 4 h. The cells were collected for surface staining of CD4 and intracellular cytokine staining. Percentages of IL-17⁺ or IFN-γ⁺ CD4⁺ T cells are shown in representative dot plots (**B**). IL-17⁺ IFN-γ⁻ cells are summarized in bar graphs (**C**). **D–E:** Naïve CD4⁺ T cells were cultured with anti-CD3 or anti-CD3/CD28 for 2 d (**D**), or differentiated under Th17 skewing conditions for 3 d and restimulated with plate-bound anti-CD3 for 1 d (**E**). IL-17 in the culture supernatants was assayed by ELISA. Naïve CD4⁺ T cells were pooled from 5 to 8 mice. Results are representative of three independent experiments (**C–E**, means + SD of triplicates). ***P* < 0.01 vs. WT control group.

especially severe asthma. The latter is often characterized by mixed responses of Th1, Th2 and Th17 cells, and resistant to the steroid therapy [17]. House dust mites (HDM; *Dermatophagoides* sp.) are one of the commonest aeroallergens worldwide and account for up to 85% of asthmatics [21, 22]. In order to delineate the pathogenesis of asthma, our laboratory has recently established a murine HDM-induced asthma model [23], besides classical ovalbumin-induced models [12–14]. Th2 and eosinophils are dominant cells in the ovalbumin-model; whereas, Th17 and neutrophils are key responders in HDM-model, mimicking severe asthma [19, 23, 24].

The protein kinase C (PKC) family of isozymes is a group of phospholipid-dependent serine/threonine kinases. There are three subfamilies: classical, novel, and atypical PKCs in this large protein family [25]. PKC isozymes exert regulatory roles to a wide range of cellular functions, such as the activation of signal transduction pathways and ion fluxes, secretion of cytokine and chemokine, cell cycle control, proliferation and differentiation, and tumorigenesis. PKC has become the focus of intense interest as a target for therapeutic intervention in a range of different diseases [26, 27]. Atypical PKC subfamily consists of two members, PKCζ and PKCλ_i, both containing Phox and Bem1 (PB1)

domain, a recently identified novel protein-protein interaction module [28]. The PB1 domain-containing signaling molecules, which include p62, par-6 and NBR1 besides atypical PKCs, play key roles in a wide range of biological processes from development to allergy and cancer [13, 29]. We have reported that PKCλ_i plays an important role in Th2 cell differentiation and T cell polarity [12]. However, the role of PKCλ_i for Th17 cells remains elusive.

In this study, we explored the role of PKCλ_i in Th17 differentiation and function in the context of ex vivo cell culture systems and an in vivo murine model of HDM-triggered allergic airway inflammation with the use of activated T cell-specific conditional PKCλ_i-deficient mice. Our data demonstrate that PKCλ_i is one of the novel regulators for Th17-differentiation and function.

2. Materials and methods

2.1. Generation of conditional PKC λ_i-deficient mice

PKCλ_i^{flx/flx} mice in C57BL/6 background were generated by a targeting strategy designed to delete *Pkcλ_i* exon 2 by the Nanjing

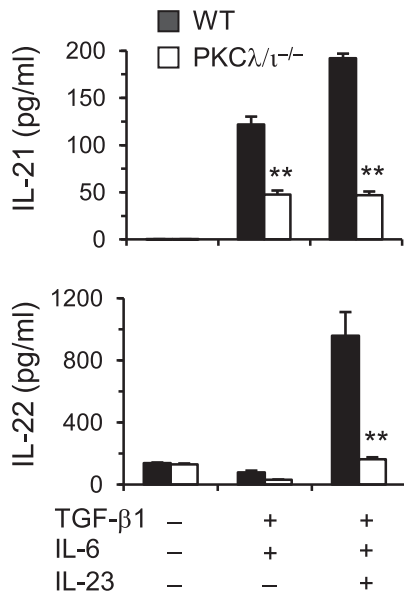


Fig. 2. IL-21 and IL-22 secretion by Th17 cells are downregulated from conditional PKCλⁱ/i^{-/-} mice. Purified naïve splenic CD4⁺ T cells were differentiated under Th17 conditions for 3 d and restimulated with plate-bound anti-CD3 for 1 d. IL-21 and IL-22 in the culture supernatants were assayed by ELISA (mean + SD of triplicates). Similar results were obtained from three independent experiments. ***P* < 0.01 vs. WT control group.

Biomedical Research Institute of Nanjing University (Nanjing, China) (Fig. S1A–B). Briefly, BAC (F-factor-based bacterial artificial chromosome) clones contain targeted gene were purchased from Invitrogen (Carlsbad, CA). Targeted gene including homologous arms was retrieved from the Bac-to-Bac (BAC) vector. *Pkcλ/i* exon 2 was flanked by loxP-FRT-neomycin-FRT cassette (Fig. S1C). The linearized targeting

construct was transfected into embryonic stem (BLU ES) cell line by standard electroporation. The recombinant clones identified by long range PCR and Southern blot analysis. Three positive ES clones were selected for injection into embryos of C57BL/6J, which were then reimplanted into OF1 pseudopregnant females. Total 14 mice were generated with chimeric rate > 50%. Mice with high chimeric rate were mated with C57BL/6 mice to generate heterozygous F1 mice (PKCλⁱ/i^{flx/wt}). The loxP-FRT-neomycin-FRT cassette was further removed by crossing the floxed mice with Flpe expressing strains and backcrossing with C57BL/6 mice to get heterozygous PKCλⁱ/i^{flx/wt} mice. After interbreeding, homozygous F2 mice (PKCλⁱ/i^{flx/flx}) were generated. By this way, we got PKCλⁱ/i^{flx/flx} mice in pure C57BL/6 background and achieved complete deletion of neomycin resistant gene. To generate conditional PKCλⁱ-deficient mice on activated T cells, PKCλⁱ/i^{flx/flx} mice were crossed with OX40^{Cre+} mice [30]. OX40 is selectively expressed on activated CD4⁺ T cells [12]. Mouse genotyping was performed by PCR using primers for PKCλⁱ-flx, OX40-Cre, and PKCλⁱ deletion to screen for homozygous conditional PKCλⁱ/i^{-/-} knockout (PKCλⁱ/i^{flx/flx}OX40^{Cre+}, hereafter referred to as PKCλⁱ/i^{-/-}) and wild-type control (PKCλⁱ/i^{flx/flx}OX40^{Cre-}, WT) mice (Fig. S1D). Age- (9–12 weeks) and sex-matched conditional PKCλⁱ-deficient mice and their WT littermates were used in each experiment.

The care and handling of the animals in this study were in strict accordance with the Guidelines for the Care and Use of Laboratory Animals at the Jiangsu Institute of Parasitic Disease (JIPD). This study was prospectively approved by the Institutional Animal Care and Use Committee of JIPD (Permit Number: [2013] 016). All efforts were made to minimize animal suffering including the use of anesthesia (sodium pentobarbital, 50 mg/kg, intraperitoneally) for the administration of HDM. Mice were monitored daily by the staff of the animal facility. At the experimental endpoint, they were euthanized under excess isoflurane anesthesia followed by cervical dislocation according to institutional guidelines.

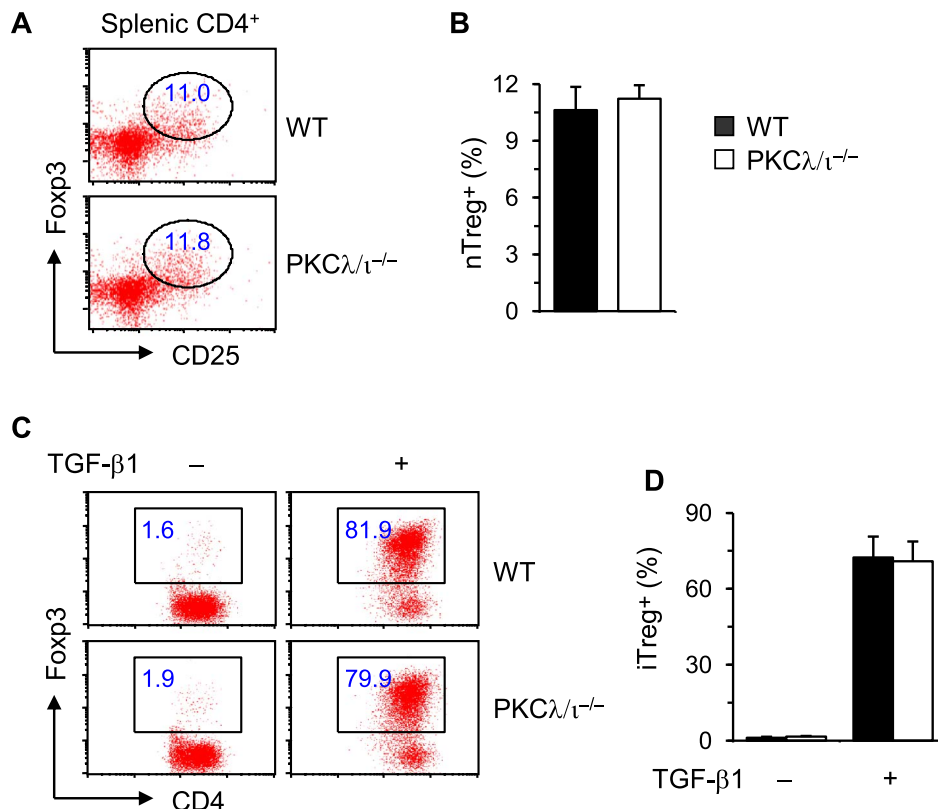


Fig. 3. Treg cells are unaffected by PKCλⁱ-deficiency. A–B: Total spleen cells were processed for Treg staining by FACS. Percentages of natural Treg (nTreg, Foxp3⁺CD25⁺) gated on splenic CD4⁺ T cells are shown in representative dot plots (A) and summarized in bar graphs (B) (n = 6–8). C–D: Purified naïve CD4⁺ T cells were differentiated under iTreg skewing conditions with TGF-β1 for 3 d and restimulated with PMA plus ionomycin for 4 h. Percentages of induced Treg (iTreg) cells are shown in representative dot plots (C) and summarized in bar graphs (D). Results are representative of two independent experiments (means + SD of triplicates).

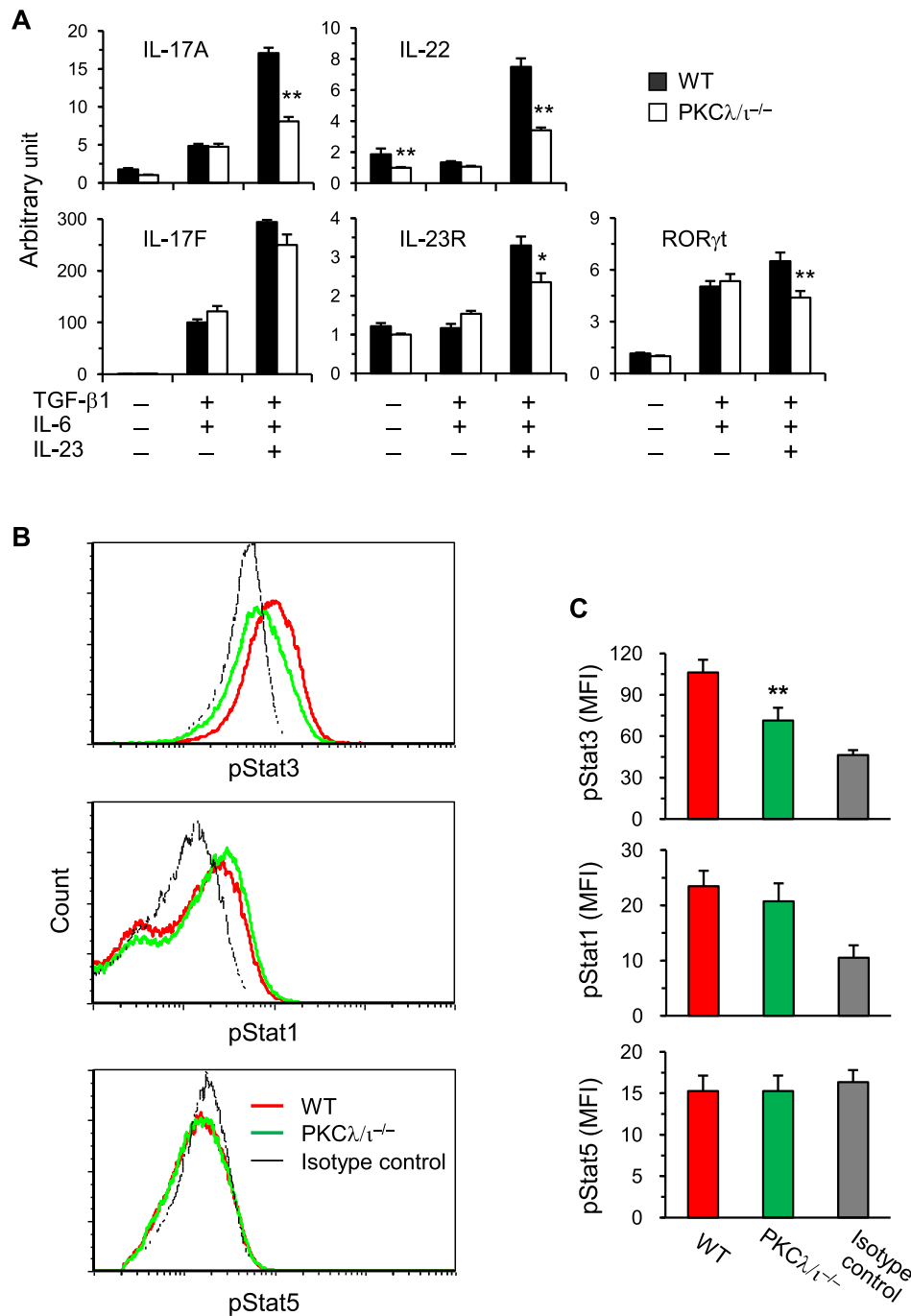


Fig. 4. Downregulation of Th17 transcription factors by loss of PKCλ/ι. **A:** Naïve splenic CD4⁺ T cells were differentiated under Th17 conditions for 3 d and restimulated with PMA plus ionomycin for 4 h. Cells were harvested to extract total RNA for real-time PCR analysis. The mRNA levels of various molecules are normalized to 18S reference and expressed as arbitrary unit. **B–C:** Naïve splenic CD4⁺ T cells were differentiated under Th17 conditions for 3 d. Cells were harvested and processed for intracellular FACS staining for pStat3 (pY705), pStat1 (pY701), and pStat5 (pY694), following BD Bioscience's protocols. Representative histograms (**B**) and the mean fluorescence intensity (MFI) (**C**) are shown. Naïve CD4⁺ T cells were pooled from 5 to 6 mice. Results (means + SD of triplicates) are representative of three (**A**) or two (**B–C**) independent experiments. **P* < 0.05, ***P* < 0.01 vs. WT group.

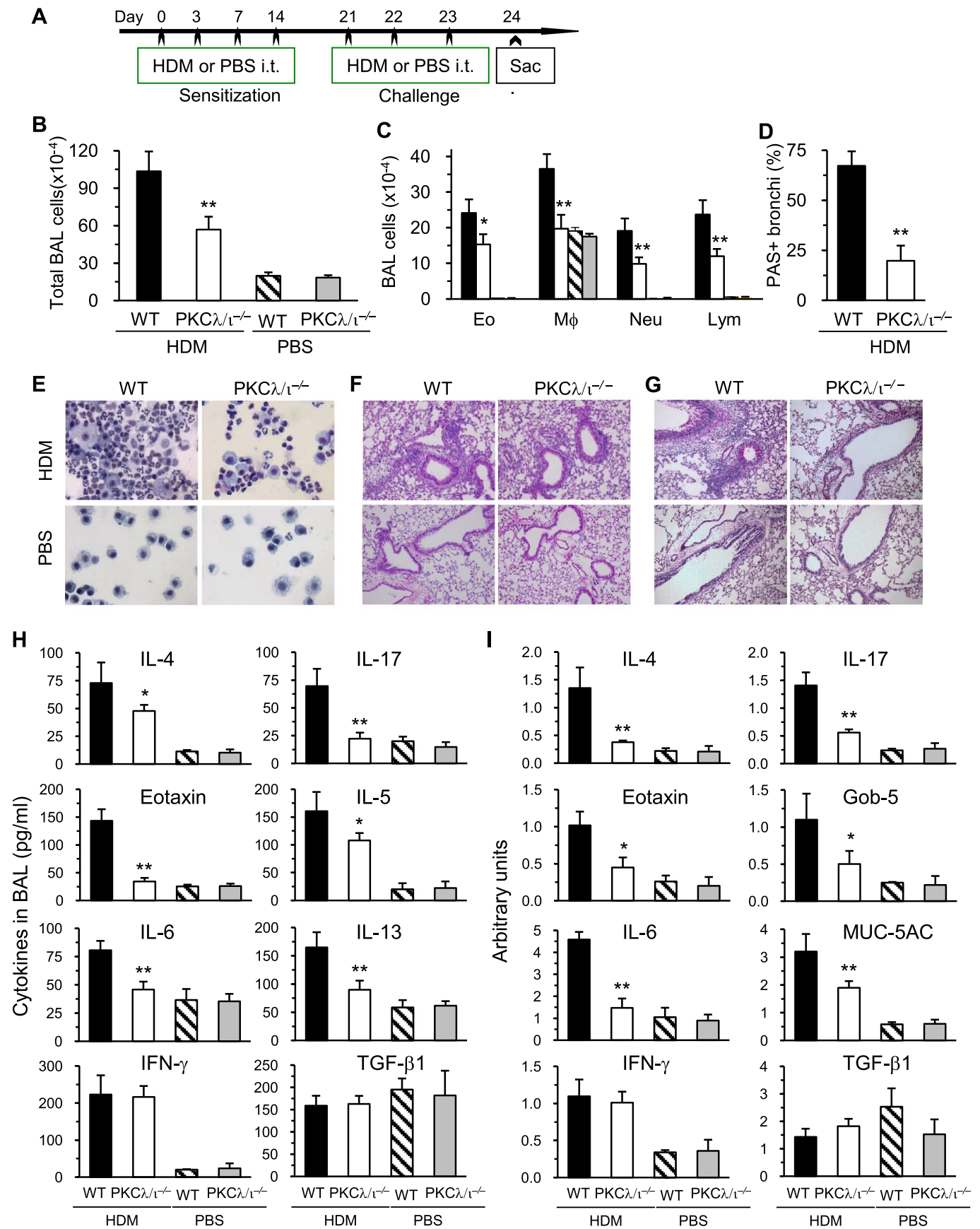
2.2. T cell activation and differentiation

Magnetically isolated naïve CD4⁺ T cells (CD62L^{hi}CD44^{lo}) by Auto MACS Pro (Miltenyi Biotec, Auburn, CA) were used for T cell activation and differentiation. T cells were activated with plate-bound anti-CD3 (10 µg/ml) plus soluble anti-CD28 (2 µg/ml) (BD Bioscience, San Jose, CA). For T cell differentiation, CD4⁺ T cells were differentiated into Th1, or Th2 cells as previously reported [12, 13, 31]. For Treg or Th17 differentiation, CD4⁺ T cells were stimulated by anti-CD3/CD28 for 3 d in the presence of TGF-β1 (5 ng/ml, for Treg), or TGF-β1 plus IL-6 (10 ng/ml, for Th17) (R&D Systems, Minneapolis, MN). In some experiments, IL-23 (10 ng/ml) (R&D Systems) was added in Th17 skewing cultures. Cells were restimulated with PMA (25 ng/ml) plus ionomycin (500 ng/ml) (Sigma, St Louis, MO) for 5 h with GolgiStop (BD Bioscience) in the last 2 h for intracellular cytokine staining; or with

plate-bound anti-CD3 (10 µg/ml) for one day for cytokine assays by ELISA in the culture supernatants.

2.3. Flow cytometry

Cells were incubated with anti-CD16/32 (2.4G2) (BD Bioscience) to block FcγR II/III, and then stained with various conjugated antibodies as indicated. BD Cytofix/Cytoperm kit or Perm buffer III (BD Bioscience) was used for intracellular cytokine or Stat staining, respectively. Stained cells were analyzed by FACSVerse with FACS Suite (BD Bioscience) or FCS Express (De Novo Software, Los Angeles, CA) software.



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Fig. 5. PKC λ / ι -deficiency inhibits HDM-induced allergic airway inflammation. WT and PKC λ / ι ^{-/-} mice were inoculated and challenged intratracheally (i.t.) with HDM to induce allergic airway inflammation (A). Control groups were inoculated i.t. with PBS alone. Mice were sacrificed 24 h after the last challenge. B–C: Quantification of total BAL cells (B), eosinophils (Eo), macrophages (M ϕ), neutrophils (Neu), and lymphocytes (Lym) (C) in BAL fluids. D–G: Representative Kwik-Diff staining for BAL cytopspins (E), H&E (F) and PAS (G) staining of lung tissue sections. Percentages of PAS stained bronchi are summarized (D). H: Cytokine/chemokine levels in BAL fluids determined by ELISA. I: mRNA levels of cytokines and chemokines in lung tissues determined by real-time PCR. Data are normalized to an 18S reference and expressed as arbitrary units. Results (means + SE, n = 8–10) are representative of three independent experiments. **P* < 0.05, ***P* < 0.01 vs. HDM-challenged WT groups.

2.4. Cytokine assay

Cytokines in the culture supernatants and BAL fluids were measured by ELISA. IL-4, IL-5 and IFN- γ were measured with OptEIA kits (BD Bioscience); IL-6, IL-13, IL-17, IL-21, IL-22 and eotaxin were measured with DuoSet ELISA kits (R&D Systems). TGF- β 1 was assayed by TGF- β 1 E_{max} ImmunoAssay System (Promega, Madison, WI). ELISA plates were developed with TMB substrate (BD Bioscience), and read with a microplate reader. Cytokine mRNA levels were measured by real-time quantitative PCR.

2.5. Real-time PCR

Total RNA was extracted from lung tissues or from cultured cells with the RNeasy Mini Kit (Qiagen, Valencia, CA), and cDNA was prepared by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed with the Platinum SYBR Green qPCR SuperMix-UDG w/RO or TaqMan Gene Expression Master Mix (Life Technologies, Carlsbad, CA) on a Light Cycler 480 II (Roche, Indianapolis, IN). The data were normalized to the 18S reference. Primers were designed with OLIG 4.0 software as reported [13].

2.6. Western Blot

Cells were either untreated or stimulated with anti-CD3/CD28 for 2 d. Cells were extracted with RIPA lysis buffer (1 $\times$ PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenyl methyl sulfonyl fluoride, and protease inhibitors). Lysates were resolved by SDS-PAGE, then electrophoretically transferred onto a nitrocellulose membrane (GE Healthcare, Piscataway, NJ) and incubated with the antibodies against PKC λ / ι (BD Bioscience) or β -actin (AC-15) (Sigma). The bands were visualized with the enhanced chemiluminescence system (Thermo Scientific, Rockford, IL).

2.7. HDM-induced allergic airway inflammation

The murine airway hyperresponsiveness was induced according to our recent report [23]. Briefly, mice of both genotypes were inoculated intratracheally (i.t.) with 15 μ g of house dust mite (HDM, Stallergenes Greer, Lenoir, NC) in 40 μ l PBS on day 0, 3, 7 and 14, once a day. Control mice were inoculated i.t. with PBS alone. Mice were challenged i.t. with HDM or PBS three times from day 21 to 23, and sacrificed 24 h after the last challenge. Bronchoalveolar lavage (BAL) fluid was aspirated and centrifuged. Total cells in the pellet were counted by using a hemacytometer. Differential cell counts on > 400 cells were performed on cytopspins stained with Shandon Kwik-Diff Stain kit (Thermo Scientific). The BAL fluid from each mouse was concentrated to 0.5 ml by centrifugation with an Amicon Ultra-4 filter unit (Millipore, Billerica, MA) for determination of cytokines by ELISA. For lung histology, the lower lobe of the right lung was fixed with 4% paraformaldehyde overnight, dehydrated, embedded in paraffin, cut into 4 μ m sections, and processed for hematoxylin/eosin (H&E) and Periodic acid–Schiff (PAS) staining. Lung tissue mRNA was analyzed by Real-time PCR.

2.8. Statistical analysis

All experimental data were analyzed and compared for statistically significant differences using two-tailed Student's *t* or Mann-Whitney *U*

test; ANOVA was used for comparing 3 or more groups. A *P* value of < 0.05 was considered significant.

3. Results

3.1. PKC λ / ι -deficiency affects Th17 differentiation and cytokine secretion

We examine the expression of PKC λ / ι in resting and activated CD4⁺ T cells. PKC λ / ι expression was highly induced in WT CD4⁺ T cells upon TCR stimulation with anti-CD3/CD28. Whereas, this induction was mostly abolished in PKC λ / ι -deficient CD4⁺ T cells (Fig. 1A).

To investigate the effect of PKC λ / ι on Th17 differentiation, purified naïve splenic CD4⁺ T cells from both WT and conditional PKC λ / ι -deficient mice were cultured in vitro under non-polarizing, Treg or Th17 polarizing conditions. By intracellular cytokine staining, IL-17 (also called IL-17A) secreting cells were significantly reduced in CD4⁺ T cells under Th17 conditions from PKC λ / ι ^{-/-} mice versus WT controls (Fig. 1B–C). Consistent with this, IL-17 levels in the culture supernatants were also markedly reduced under non-skewing and Th17-skewing conditions from PKC λ / ι -deficient T cells (Fig. 1D–E).

IL-21 and IL-22 are important Th17 effector cytokines. IL-21 is secreted by Th17 and other cells, which has autocrine effect to promote Th17 maturation [32]. The secretion of IL-22 from Th17 cells needs the presence of IL-23 to induce the full maturation of Th17 cells [33]. These two cytokines, IL-21 and IL-22, were also significantly reduced from PKC λ / ι -deficient Th17 cells (Fig. 2).

On the other hand, PKC λ / ι -deficiency did not affect Treg cells. The natural Treg (nTreg) in the spleens were similar between WT and PKC λ / ι ^{-/-} mice (Fig. 3A–B). Under Treg skewing cultures conditions of CD4⁺ T cells, percentages of induced Treg (iTreg) cells did not alter by the loss of PKC λ / ι (Fig. 3C–D). Taken together, PKC λ / ι -deficiency significantly inhibited Th17 differentiation and effector cytokine secretion, without affecting Treg cells.

3.2. Underlying molecular mechanisms whereby PKC λ / ι -deficiency affects Th17 cells

To begin to understand how PKC λ / ι regulates Th17 cells, the transcription factors and receptors related to Th17-differentiation were determined by real-time PCR under Th17 polarizing condition in vitro. Ror γ t, specific Th17 transcription factor, was found downregulated in PKC λ / ι -deficient T cells (Fig. 4A). Consistent with that, the mRNA levels of Th17 effector cytokines, IL-17A, IL-17F and IL-22, were downregulated in Th17-skewing conditions in the presence of IL-23, although the change of IL-17F was not so significantly. Moreover, the expression of IL-23R was significantly inhibited by the loss of PKC λ / ι (Fig. 4A).

IL-6 and IL-23 induce Stat3 phosphorylation to promote Th17 differentiation or full maturation. The level of phospho-Stat3 (pStat3) was markedly increased from WT CD4⁺ T cells in Th17-skewing culture conditions. This upregulation of pStat3 was significantly impaired with the loss of PKC λ / ι (Fig. 4B–C). On the contrast, the expression of pStat1 and pStat5, key transcription factors for Th1 and Treg cells respectively, did not alter significantly (Fig. 3B–C). These data suggest that PKC λ / ι may regulate Th17 cells at least in part via transcription factors Ror γ t and Stat-3. More thorough investigation is clearly necessary to better define how PKC λ / ι regulates Th17 cells.

3.3. PKC λ /i-deficiency inhibits HDM-induced allergic airway inflammation

As PKC λ /i can regulate Th17 differentiation and cytokine production in vitro, it is possible that there may be particular pathways set in motion in vivo that require PKC λ /i for an adequate Th17 function. For this reason, we next investigated whether PKC λ /i was necessary for an optimal Th17 response in vivo. We developed a murine model of HDM-induced allergic airway inflammation (Fig. 5A), in which Th17 cells and neutrophils are dominant responder among others [23]. In the next series of experiments, we determined the requirement for PKC λ /i with the use of this model.

Total BAL cells increased over fivefold in WT mice intratracheally inoculated HDM compared to PBS-inoculated ones (Fig. 5B). However, there was a dramatic reduction in BAL cells, including eosinophils and neutrophils in PKC λ /i-deficient mice with HDM inoculation (Fig. 5B–C, E). As revealed by lung histological analysis, a mixed cell infiltration, including eosinophils, macrophages, neutrophils and lymphocytes were observed around blood vessels and bronchi in the lungs of HDM-inoculated WT mice. This massive infiltration of inflammatory cells was markedly inhibited in PKC λ /i^{−/−} mice (Fig. 5F). We thought that the inhibitory effect of PKC λ /i-deficiency on allergic airway inflammation was due to reduced Th2 and Th17 cytokine secretion from conditional PKC λ /i^{−/−} mice. Consistent with this notion, Th2 effector cytokine IL-4, IL-5, IL-6, and Th17 cytokine IL-17 in BAL fluids were significantly reduced from PKC λ /i^{−/−} mice as compared to that from WT littermates in response to HDM challenge (Fig. 5H). The mRNA levels of Th2 and Th17 cytokines in lungs followed similar patterns as protein levels in BAL (Fig. 5I). However, TGF- β 1 and Th1 cytokine IFN- γ were not affected by the loss of PKC λ /i at both protein and mRNA levels (Fig. 5H–I).

In additions, eotaxin, an important non-T cell chemokine, selectively recruit eosinophils from the airway microvessels into the lung tissue, which plays a key role in the onset of allergic airway inflammation [34]. Eotaxin was markedly inhibited in BAL fluids. Its mRNA level in lungs was also down-regulated from PKC λ /i^{−/−} mice. Mucus hypersecretion is important for the pathogenesis and severity of asthma, which was significantly downregulated in HDM-inoculated PKC λ /i^{−/−} mice compared to their WT littermates as demonstrated by PAS staining (Fig. 5D, G). In PBS-inoculated control groups, no PAS stained bronchus was seen in both genotypes. Gob-5 gene has been suggested to promote the synthesis of mucin glycoproteins, the main proteins in mucus, by upregulation of MUC-5AC gene expression [12, 35]. The expression of both Gob-5 and MUC-5AC was inhibited in lungs in PKC λ /i^{−/−} mice (Fig. 5I). These data indicate that PKC λ /i not only controls Th17 in vitro differentiation and cytokine secretion, it may significantly affect in vivo Th17 function in allergic airway inflammation.

4. Discussion

We have previously reported that PKC λ /i regulates Th2 differentiation and function in the context of in vitro cell culture system and in vivo allergic airway inflammation [12]. PKC λ /i emerges as an important component of the signaling cascades leading to Th2 cytokine production and asthma. However, whether PKC λ /i controls Th17 cells remains elusive.

In this study, we investigated the potential role of PKC λ /i on Th17 differentiation and function with the use of newly established PKC λ /i conditional knockout mice. Our findings indicated that PKC λ /i-deficiency affected not only Th17 differentiation and cytokine secretion in vitro, but also HDM-induced allergic airway inflammation. HDM is a common indoor antigen and potent inducer of allergic asthma [36, 37]. We developed a murine asthma model induced by repeated intracheal inoculation of HDM [23]. This model mimics clinical severe asthma, in which Th17 and neutrophils are dominant responders [17, 38]. In this model, the types of infiltrative cells and Th responses may differ

according to mouse strains, immunization regimens, such as times, dosages and routes of inoculation, etc. In our current study, mixed inflammatory cells, including eosinophils, neutrophils, macrophages, lymphocytes were observed in WT mice. These findings were consistent with other investigators' reports, in which Th17 and neutrophils were key infiltrating cells among others in the lungs [39–41]. In conditional PKC λ /i knockout mice, however, we found a significant inhibition of infiltrating cells, including neutrophils in the lungs and airways. IL-17 in BAL fluids and its mRNA expression in the lungs were significantly downregulated. These data suggest that PKC λ /i may regulate Th17 differentiation and function both in vitro and in vivo.

The underlying molecular mechanisms how PKC λ /i controls Th17 cells remain elusive. We have reported that PKC λ /i-deficiency affected activation-induced T cell polarity, a more general cellular function related to growth and activation. PKC λ /i regulates Th2 cells, at least in part, through the control of T cell polarity [12]. Whether T cell polarity links to Th17-differentiation is yet to be clarified. Another possibility is that PKC λ /i could regulate Th17-related transcription factors and key molecules. Our previous report demonstrated that activation of the transcription factors, such as NF- κ B and nuclear factor of activated T-cells (NFAT), were impaired in PKC λ /i-deficient activated T cells [12]. In current study, we found that Stat3 and Ror γ t, important transcription factors for IL-6 pathway and Th17 differentiation respectively, were downregulated in PKC λ /i-mutant CD4⁺ T cells upon stimulation. IL-23 is important for Th17 full maturation and function [33]. Its receptor IL-23R on Th17 cells were also reduced in PKC λ /i-mutant CD4⁺ T cells. More in depth investigation is obviously necessary to fully elucidate the underlying mechanisms.

In summary, we have previously reported that PKC λ /i-deficiency affected Th2 differentiation and function. In this study, we demonstrated that PKC λ /i was important for Th17-differentiation and function. Loss of PKC λ /i in CD4⁺ T cells led to impaired Th17-differentiation and cytokine secretion in vitro. PKC λ /i-deficient mice showed attenuated allergic inflammation as characterized by inhibited cellular infiltration and reduced Th2 as well as Th17 effector cytokines in the airways. The underlying molecular mechanisms are yet to be elucidated. Our data support the notion that the impaired Th17 differentiation and function by PKC λ /i-deficiency, at least in part, related to the downregulation of Th17 key transcription factors, such as Stat3 and Ror γ t. More in depth investigations are clearly needed to elucidate the underlying cellular and molecular mechanisms.

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Transparency document

The Transparency document associated with this article can be found in online version.

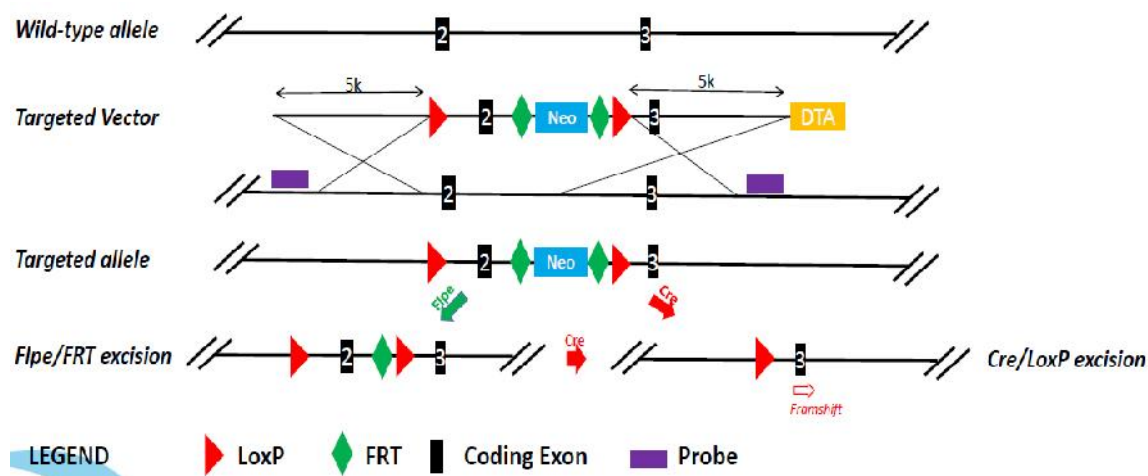
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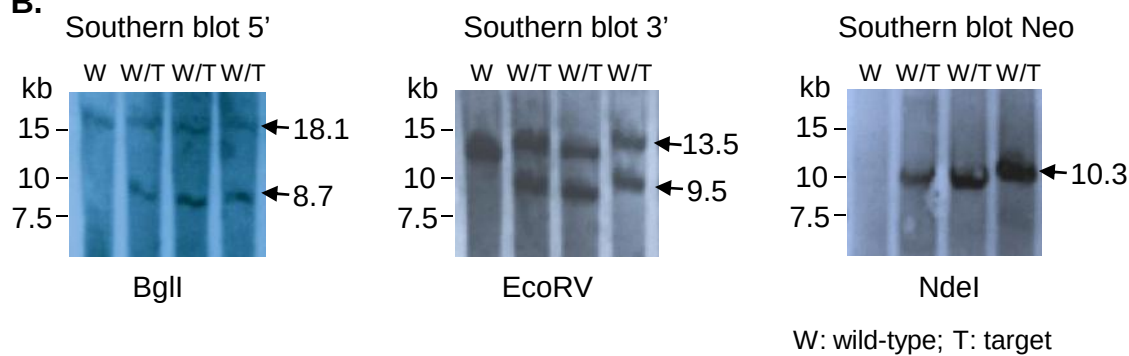
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Fig. S1

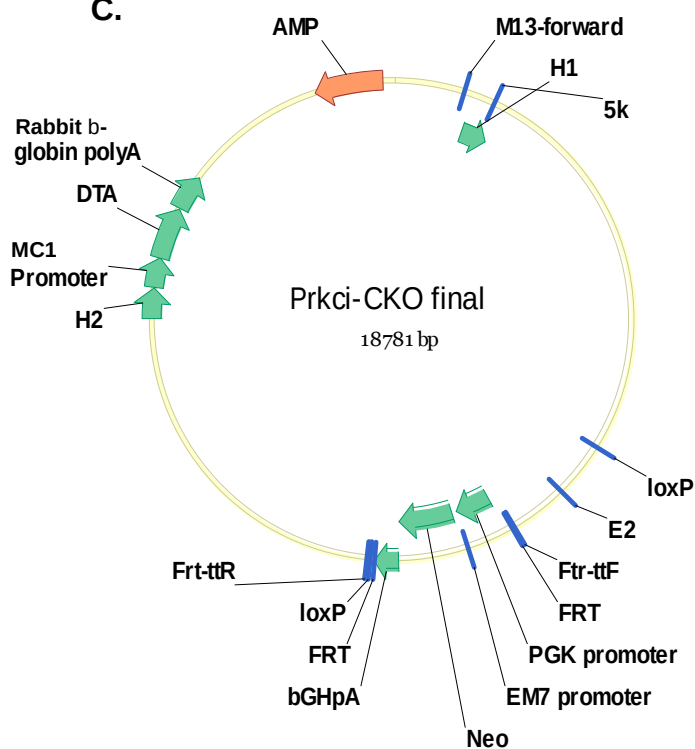
A.



B.



C.



D.

