

ORIGINAL ARTICLE

Experimental Models of Allergic Disease

Rational targeting Cdc42 restrains Th2 cell differentiation and prevents allergic airway inflammation

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Summary

Background: Asthma is an allergic airway inflammation-driven disease that affects more than 300 million people world-wide. Targeted therapies for asthma are largely lacking. Although asthma symptoms can be prevented from worsening, asthma development cannot be prevented. Cdc42 GTPase has been shown to regulate actin cytoskeleton, cell proliferation and survival.

Objectives: To investigate the role and targeting of Cdc42 in Th2 cell differentiation and Th2-mediated allergic airway inflammation.

Methods: Post-thymic Cdc42-deficient mice were generated by crossing Cdc42^{flox/flox} mice with dLck^{icre} transgenic mice in which Cre expression is driven by distal Lck promoter. Effects of post-thymic Cdc42 deletion and pharmacological targeting Cdc42 on Th2 cell differentiation were evaluated in vitro under Th2-polarized culture conditions. Effects of post-thymic Cdc42 deletion and pharmacological targeting Cdc42 on allergic airway inflammation were evaluated in ovalbumin- and/or house dust mite-induced mouse models of asthma.

Results: Post-thymic deletion of Cdc42 led to reduced peripheral CD8⁺ T cells and attenuated Th2 cell differentiation, with no effect on closely related Th1, Th17 and induced regulatory T (iTreg) cells. Post-thymic Cdc42 deficiency ameliorated allergic airway inflammation. The selective inhibition of Th2 cell differentiation by post-thymic deletion of Cdc42 was recapitulated by pharmacological targeting of Cdc42 with CASIN, a Cdc42 activity-specific chemical inhibitor. CASIN also alleviated allergic airway inflammation. CASIN-treated Cdc42-deficient mice showed comparable allergic airway inflammation to vehicle-treated Cdc42-deficient mice, indicative of negligible off-target effect of CASIN. CASIN had no effect on established allergic airway inflammation.

Conclusion and Clinical Relevance: Cdc42 is required for Th2 cell differentiation and allergic airway inflammation, and rational targeting Cdc42 may serve as a preventive but not therapeutic approach for asthma control.

KEYWORDS

animal models, asthma, CASIN, Cdc42, T cells, Th2 differentiation

1 | INTRODUCTION

T cells play a critical role in mediating adaptive immunity to a variety of pathogens.¹ T cells are developed in the thymus. The most immature populations in the thymus are comprised of CD4⁺CD8[−] thymocytes. CD4⁺CD8[−] thymocytes differentiate to CD4⁺CD8⁺ cells. CD4⁺CD8⁺ cells then differentiate to CD4⁺ or CD8⁺ T cells or CD4⁺Foxp3⁺ natural regulatory T cells (nTreg). CD4⁺ and CD8⁺ T cells migrate to peripheral tissues, where they are maintained as naïve CD4⁺ and CD8⁺ T cells.^{2,3}

In response to antigen stimulation, naïve T cells are activated and differentiated into effector T cells. CD4⁺ effector T cells include T helper (Th) 1, Th2 and Th17 cells.^{1,3–5} Th cells are characterized by secreting particular profiles of cytokines and exerting distinct functions in vivo. For example, Th1 cells produce IFN- γ and mediate cellular immunity against intracellular pathogens and autoimmunity.^{1,4,5} Th17 cells produce IL-17 and are important for eliminating extracellular pathogens and for autoimmunity.^{6,7} Th2 cells secrete IL-4, IL-5 and IL-13, and play a key role in humoral immunity, allergy and asthma, an allergic airway inflammation-driven disease characterized by lung eosinophilia, elevated serum immunoglobulin E (IgE), and airway hyperresponsiveness and goblet cell metaplasia.^{1,4,5,8–10} On the other hand, CD4⁺ naïve T cells can also differentiate to CD4⁺Foxp3⁺-induced regulatory T cells (iTreg) that together with nTreg, act to maintain immune tolerance by inhibition of T cell proliferation and effector T cell function.¹¹

Cdc42 of the Rho small GTPase family is an intracellular signal transducer that cycles between an inactive GDP-bound form and an active GTP-bound form.¹² Cdc42 has been shown to regulate actin cytoskeleton reorganization, cell migration, proliferation, survival and oncogenesis.^{13–16} By T cell-specific Cdc42 deletion, we have recently found that Cdc42 promotes thymocyte development, peripheral T cell homeostasis and iTreg cells but suppresses T cell activation, Th1 and Th17 cell differentiation, with no effect on Th2 cells.^{17–19}

In this study, we aimed to investigate the physiological role of Cdc42 in Th2 cell differentiation and function. We achieved post-thymic deletion of Cdc42 and found that post-thymic deletion of Cdc42 inhibited Th2 differentiation with no effect on Th1, Th17 and iTreg cells. Post-thymic Cdc42 deletion ameliorated Th2-mediated allergic airway inflammation. Pharmacological inhibition of Cdc42 with CASIN, a Cdc42 activity-specific inhibitor,²⁰ was able to recapitulate the effects of post-thymic deletion of Cdc42 on selective inhibition of Th2 differentiation and on alleviation of allergic airway inflammation. However, CASIN could not ameliorate established allergic airway inflammation. Thus, Cdc42 emerges as a critical regulator of Th2 cell differentiation and may be a preventive, but not therapeutic target, for asthma.

2 | METHODS

2.1 | Mice

Cdc42^{flox/flox} mice were generated as previously described.^{17,21} Cdc42^{flox/flox} mice were mated with dLCK^{iCre} transgenic mice, in which Cre expression is controlled by distal Lck promoter,²² to generate

Cdc42^{flox/flox}dLCK^{iCre} mice. Mouse genotyping was performed by PCR. Age- (8–10 weeks) and sex-matched mice were used in each experiment. Animals were housed under specific pathogen-free conditions in the animal facility at the Cincinnati Children's Hospital Research Foundation in compliance with the Cincinnati Children's Hospital Medical Center Animal Care and Use Committee protocols (2017-0025).

2.2 | Ovalbumin (OVA)-induced allergic airway inflammation

Allergic airway inflammation was induced as described in our previous reports.^{23–25} Briefly, mice were immunized ip with 50 μ g of OVA (Grade V; Sigma-Aldrich, St Louis, MO, USA) in 100 μ L (2 mg) of alum (Imject Alum; Pierce, IL, USA) on day 0 and day 7. On day 14, the mice were challenged two times (60 minutes each delivered 4 hours apart) with aerosolized 1% OVA dissolved in PBS by an Omron NE-C25 Nebulizer (Omron Healthcare, Bannockburn, IL, USA). On day 15, the mice were challenged one more time. For established allergic airway inflammation model, the mice were rechallenged on days 46 and 47. Control mice were challenged with PBS. Where indicated, CASIN was injected ip into the mice. Mice were killed 24 hours after the last challenge or rechallenge. Bronchoalveolar lavage (BAL) fluids were aspirated and centrifuged, and total cells in the pellet were counted by using a haemocytometer. Differential cell counts on >400 cells were performed on cytospins stained with Shandon Kwik-Diff Stain kit (Thermo Fisher Scientific, Rockford, IL, USA). The BAL fluid from each mouse was concentrated to 0.5 mL by centrifugation with an Amicon Ultra-4 filter unit (Millipore, Billerica, MA, USA) for the 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 haematoxylin/eosin (H&E) staining. Lung tissue mRNA was analysed by quantitative real-time RT-PCR. Serum levels of OVA-specific IgE were measured by ELISA with the use of biotinylated goat anti-mouse IgE (BD Bioscience, San Jose, CA, USA), as our previous report.²⁵

2.3 | House dust mite (HDM)-induced allergic airway inflammation

The murine airway hyperresponsiveness was induced according to our recent reports^{26,27} with modifications.^{28,29} Briefly, mice were inoculated intratracheally (i.t.) with 40 μ g of house dust mite (HDM; Stallergenes Greer, Lenoir, NC) in 40 μ L PBS three times a week for 5 weeks. Control mice were inoculated i.t. with PBS alone. Mice were killed 24 hours after the last inoculation. BAL fluid was aspirated and centrifuged. Total cells and differential cell counts in the pellet were performed. Cytokines in the BAL fluid were determined 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 haematoxylin/eosin (H&E) and periodic acid-Schiff (PAS) staining. Lung tissue mRNA was analysed by real-time PCR. HDM-specific IgE was

measured by ELISA with the use of biotinylated goat anti-mouse IgE (BD Bioscience), as our previous report.²⁶

2.4 | Quantitative Real-time RT-PCR

Total RNA was extracted from lung tissues or Th2 cells with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and cDNA was prepared

by using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time RT-PCR was performed with the Platinum SYBR Green qPCR SuperMix-UDG w/ RO or TaqMan Gene Expression Master Mix (Life Technologies, Carlsbad, CA, USA) on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). The data were normalized to the 18S reference. Primers were designed with OLIGO 4.0 software as reported.²⁴

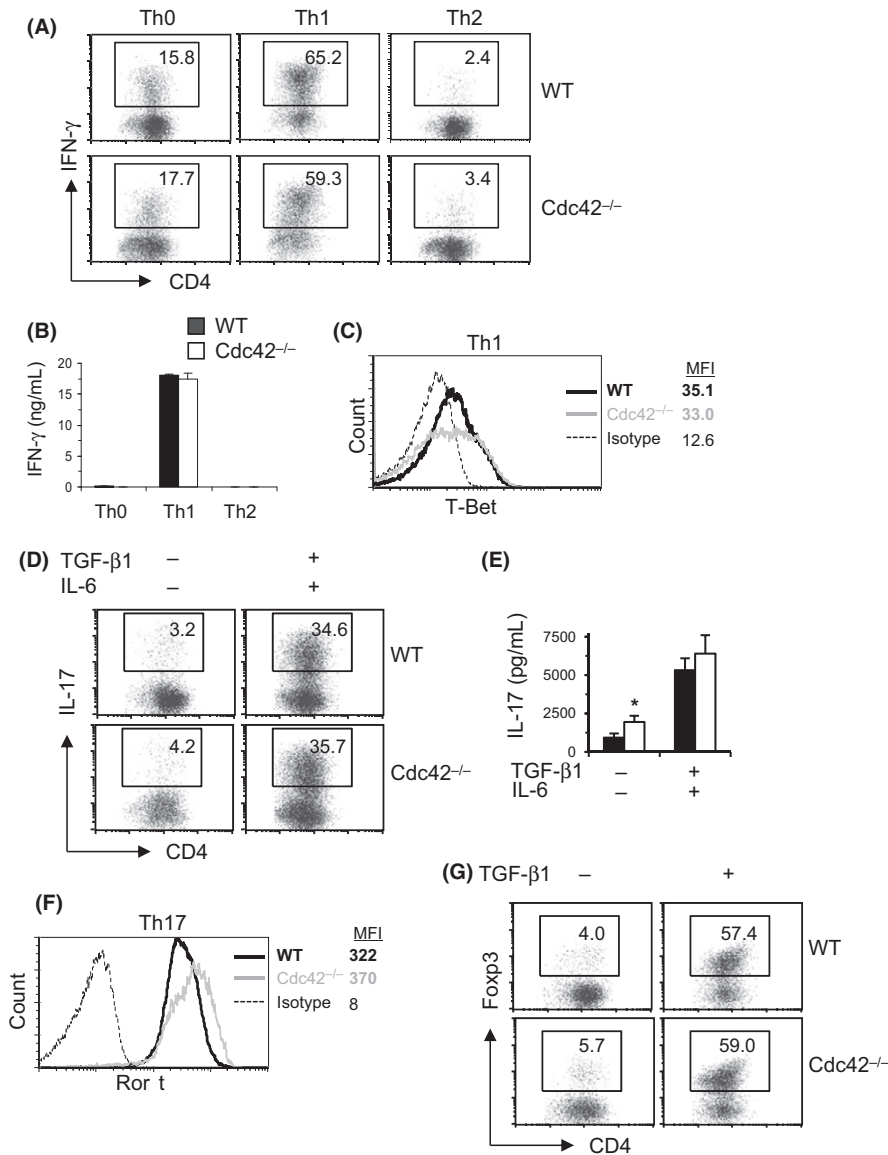


FIGURE 1 Post-thymic Cdc42 deficiency has no effect on Th1, Th17 and iTreg differentiation. A-C, Naïve CD4⁺ T cells were differentiated under Th0, Th1 or Th2 conditions for 4 days followed by PMA plus ionomycin restimulation for 5 h, in the presence or absence of BD GolgiPlugTM at the last 2 h. Cells were processed for CD4 surface staining and IFN- γ and T-Bet intracellular staining and flow cytometry analysis. Percentages of IFN- γ ⁺CD4⁺ T cells are shown in representative dot plots (A). IFN- γ in the culture supernatants was collected from other sets of cultures without BD GolgiPlugTM and assayed by ELISA (B). Representative histogram of T-Bet is shown (C). D-F, Naïve CD4⁺ T cells were differentiated under non-skewing (no TGF- β 1/IL-6) or Th17 (TGF- β + IL-6) skewing culture conditions for 4 days followed by PMA plus ionomycin restimulation. The cells were processed for surface staining of CD4 and intracellular staining of IL-17 and Ror γ t for flow cytometry analysis. Percentages of CD4⁺IL-17⁺ T cells are shown in representative dot plots (D). IL-17 in the culture supernatants was assayed by ELISA (E). Representative histogram of Ror γ t is shown (F). G, Naïve CD4⁺ T cells were differentiated under non-skewing (no TGF- β 1) or iTreg (TGF- β) skewing culture conditions for 4 days followed by PMA plus ionomycin restimulation. The cells were processed for surface staining of CD4 and intracellular staining of Foxp3 for flow cytometry analysis. Percentages of Foxp3⁺ T cells are shown in representative dot plots. Results are representative of 5-8 mice. Error bars represent SD of triplicates. *P < 0.05

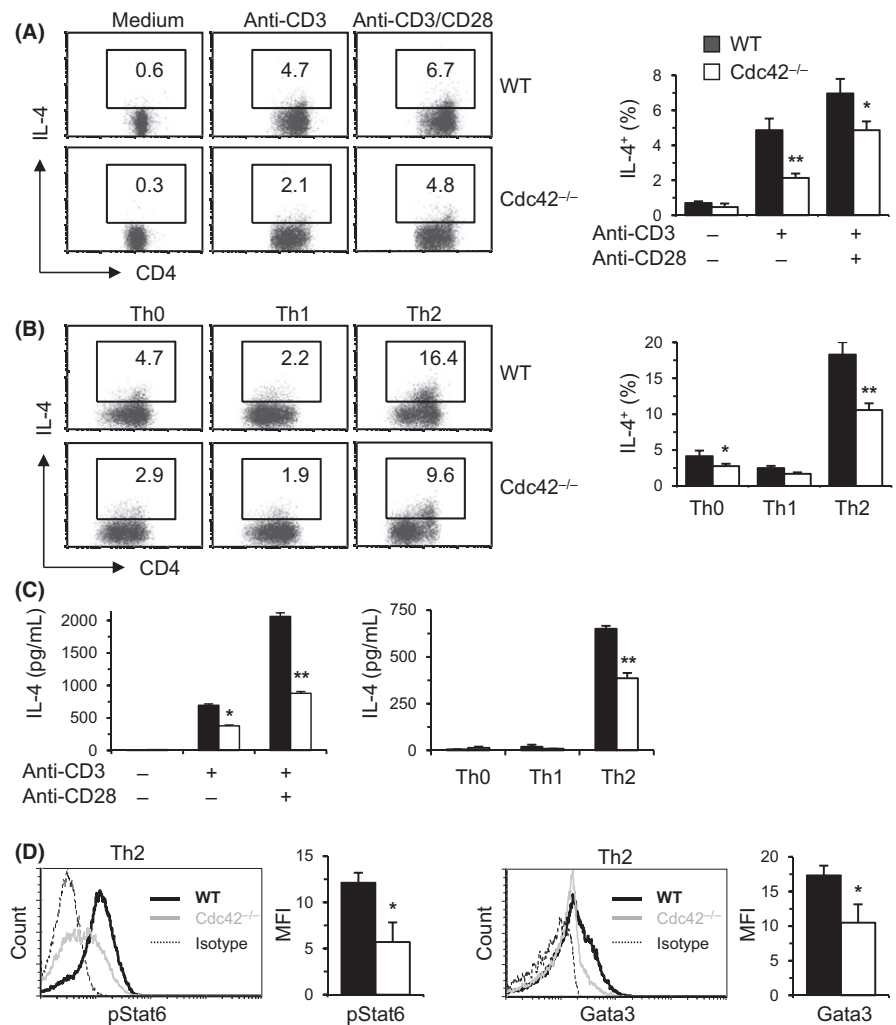


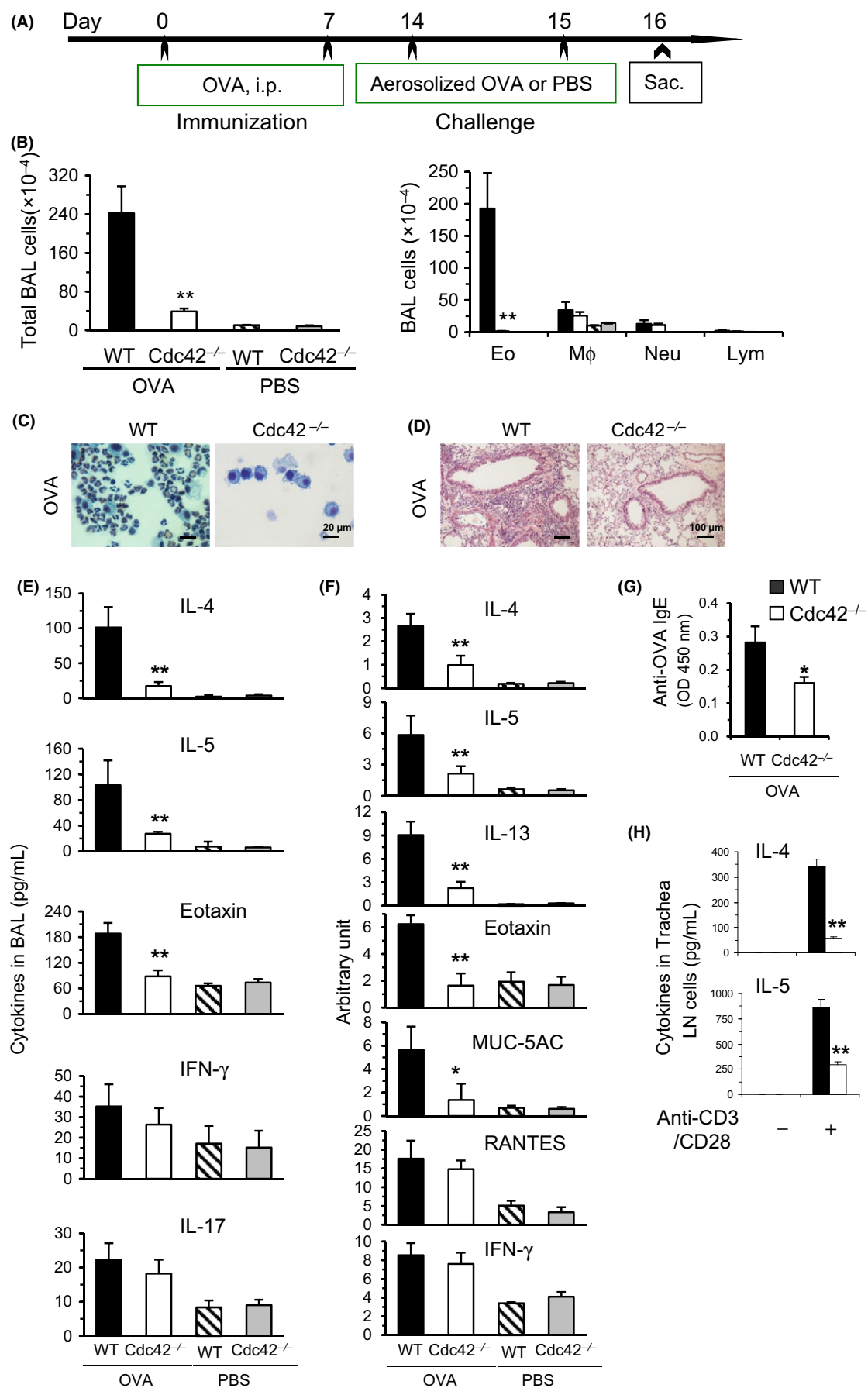
FIGURE 2 Post-thymic Cdc42 deficiency suppresses Th2 cell differentiation. Naïve CD4⁺ T cells were cultured with anti-CD3 or anti-CD3/CD28 for 2 d (A) or differentiated under Th0, Th1 or Th2 skewing conditions for 4 days and restimulated with PMA plus ionomycin for 5 h (B). The cells were collected for surface staining of CD4 and intracellular staining of IL-4. Percentages of CD4⁺IL-4⁺ T cells are shown in representative dot plots (A and B, Left) and summarized in bar graphs (A and B, Right). IL-4 in the culture supernatants was assayed by ELISA (C). The expression of pStat6 and Gata3 was detected by flow cytometry. Representative histograms are shown, and the mean fluorescence intensity (MFI) is summarized in bar graphs (D). Results are representative of 5–8 mice. Error bars represent SD of triplicates. **P* < 0.05, ***P* < 0.01

2.5 | T cell activation and differentiation

Sorted naïve T cells (CD62L^{hi}CD44^{lo}) were used for T cell activation and differentiation. Naïve T cells were activated with plate-bound anti-CD3 (10 µg/mL) plus soluble anti-CD28 (2 µg/mL) (BD Bioscience). For T cell differentiation, CD4⁺ naïve T cells were differentiated into Th0, Th1, Th2, Th17, or iTreg cells as previously reported.^{23–25,30} Briefly, CD4⁺ T cells were stimulated by anti-CD3/CD28 for 4 days with IL-2 (20 ng/mL) in the presence of anti-IFN-γ and anti-IL-4 (both 10 µg/mL, for Th0), anti-IL-4 and IL-12 (20 ng/mL, for Th1), anti-IFN-γ and IL-4 (20 ng/mL, for Th2).

For iTreg and Th17 conditions, CD4⁺ T cells were stimulated by anti-CD3/CD28 for 4 days with anti-IFN-γ and anti-IL-4 (both 10 µg/mL) in the presence of IL-2 (20 ng/mL) plus TGF-β1 (5 ng/mL, for Treg), or TGF-β1 plus IL-6 (10 ng/mL, for Th17) (all from R&D Systems, Minneapolis, MN, USA). Cells were restimulated with PMA (25 ng/mL) plus ionomycin (500 ng/mL) (Sigma, St Louis, MO, USA) for 5 hours with GolgiStop (BD Bioscience) in the last 2 hours for intracellular cytokine staining, or without GolgiStop for cytokine assays in the culture supernatants by ELISA. Where indicated, CASIN (Chembridge Corporation, San Diego, CA, USA) was added to the cultures.

FIGURE 3 Post-thymic Cdc42 deficiency inhibits OVA-induced allergic airway inflammation. A, WT and Cdc42^{-/-} mice were immunized ip with OVA and then challenged with aerosolized OVA or PBS as control. Mice were killed 24 h after the last challenge. B, Quantification of total BAL cells (Left), eosinophils (Eo), macrophages (Mφ), neutrophils (Neu) and lymphocytes (Lym) (Right) in BAL fluids. C, D, Representative Kwik-Diff staining for BAL cytopins (C, scale bar, 20 µm) and H&E staining of lung tissue sections (D, scale bar, 100 µm). E, Cytokine/chemokine levels in BAL fluids determined by ELISA. F, 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. G, OVA-specific IgE levels in serum of mice immunized and challenged with OVA. H, Cytokine levels, as determined by ELISA, in 2 d-cultured supernatants of draining trachea lymph node cells isolated from OVA-challenged mice and restimulated in vitro with anti-CD3/CD28. Results are representative of two independent experiments. Error bars represent SE. *n* = 5–8. **P* < 0.05, ***P* < 0.01 vs OVA-challenged and/or anti-CD3/CD28-restimulated WT control [Colour figure can be viewed at wileyonlinelibrary.com]



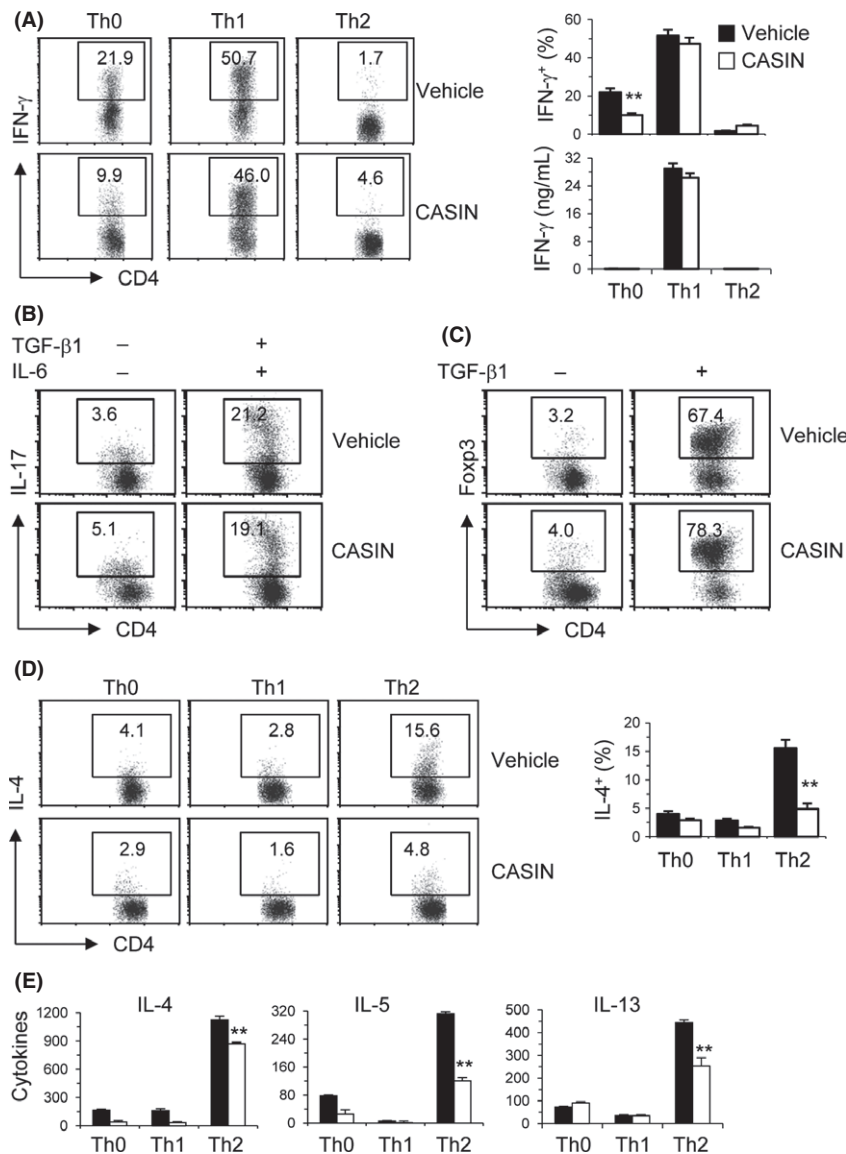
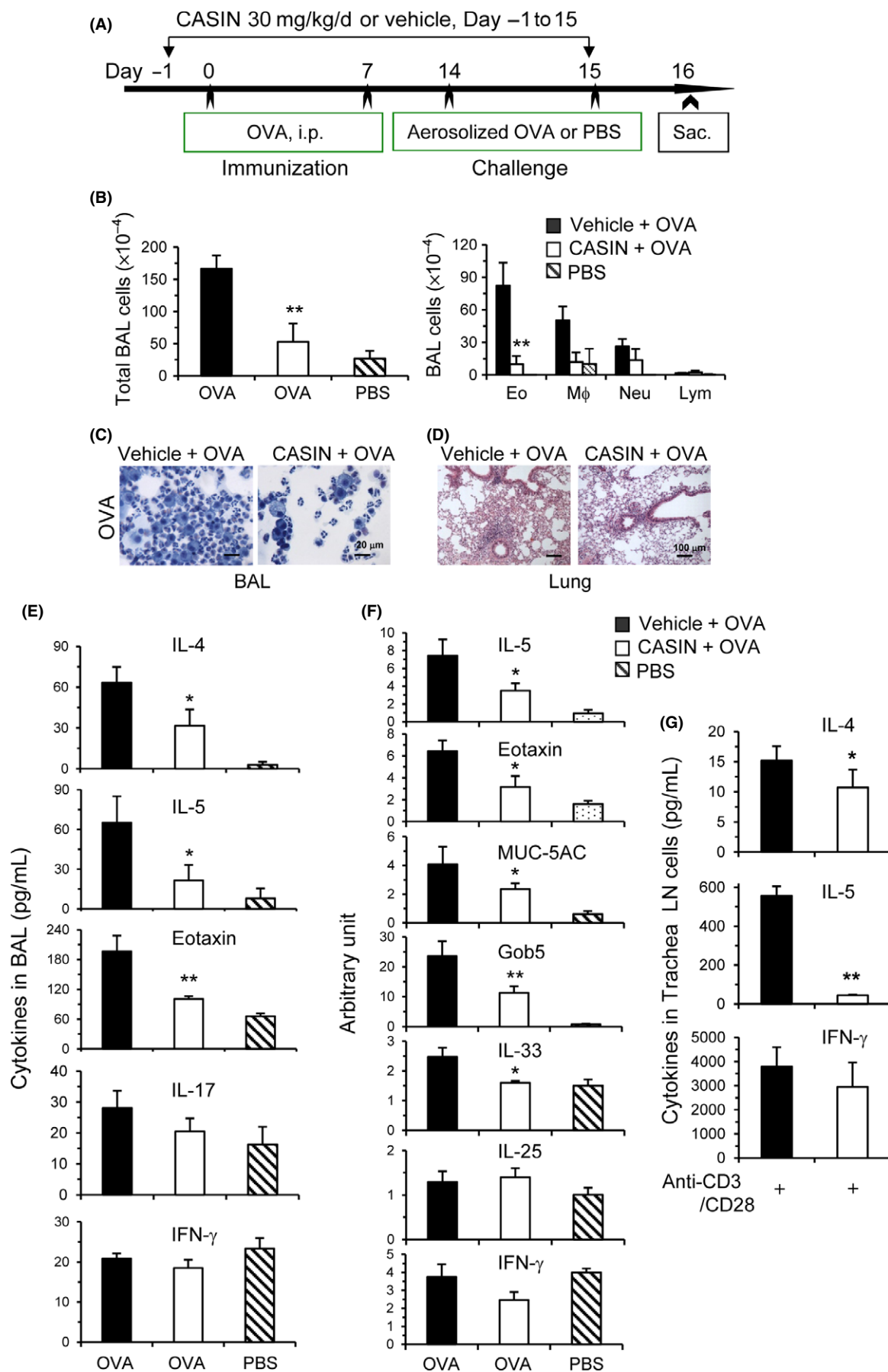


FIGURE 4 Pharmacological targeting Cdc42 by Cdc42 inhibitor CASIN selectively suppresses Th2 differentiation. A, Naïve WT CD4⁺ T cells were differentiated under Th0, Th1 or Th2 conditions for 4 days and restimulated with PMA plus ionomycin for 5 h in the absence (vehicle) or presence of CASIN (10 μ mol/L) throughout the culture. The cells were processed for IFN- γ intracellular staining. Percentages of CD4⁺IFN- γ ⁺ T cells are shown in representative dot plots (A, Left) and summarized in bar graph (A, Upper Right). IFN- γ in the culture supernatants was assayed by ELISA (A, Lower Right). B, Naïve WT CD4⁺ T cells were differentiated under non-skewing or Th17 (TGF- β + IL-6) skewing conditions for 4 days and restimulated with PMA plus ionomycin in the absence (vehicle) or presence of CASIN (10 μ mol/L) throughout the culture. The cells were processed for CD4 surface staining and IL-17 intracellular staining. Percentages of CD4⁺IL-17⁺ cells are shown in representative dot plots. C, Naïve WT CD4⁺ T cells were differentiated under non-skewing or iTreg (TGF- β) skewing conditions for 4 days and restimulated with PMA plus ionomycin in the absence (vehicle) or presence of CASIN (10 μ mol/L) throughout the culture. The cells were processed for CD4 surface staining and Foxp3 intracellular staining. Percentages of CD4⁺Foxp3⁺ cells are shown in representative dot plots. D, E, Naïve WT CD4⁺ T cells were differentiated under Th0, Th1, or Th2 skewing conditions for 4 days and restimulated with PMA plus ionomycin for 5 h in the absence (vehicle) or presence of CASIN (10 μ mol/L) throughout the culture. Cells were processed for CD4 surface staining and IL-4 intracellular staining. Percentages of CD4⁺IL-4⁺ cells are shown in representative dot plots and summarized in bar graph (D). Th2 cytokines in the culture supernatants were assayed by ELISA (E). Results are representative of two independent experiments. Error bars represent SD of triplicates. ** P < 0.01

FIGURE 5 CASIN alleviates allergic airway inflammation. WT mice were injected ip daily with CASIN (30 mg/kg) or vehicle, starting 1 day before OVA immunization until 1 day before killing (A). Total BAL cells and differential cell counts (B), representative Kwik-Diff staining for BAL cytopins (C), H&E staining of lung tissue sections (D), cytokine and eotaxin levels in BAL fluids (E) and mRNA levels of cytokines and chemokines in lung tissues determined by real-time PCR (F) are shown. Draining trachea lymph node cells were isolated from OVA-challenged mice and restimulated in vitro with anti-CD3/CD28 for 2 days. Cytokine levels were then determined by ELISA (G). Results are representative of two independent experiments. Error bars represent SE. n = 6-8. * P < 0.05, ** P < 0.01 vs vehicle [Colour figure can be viewed at wileyonlinelibrary.com]



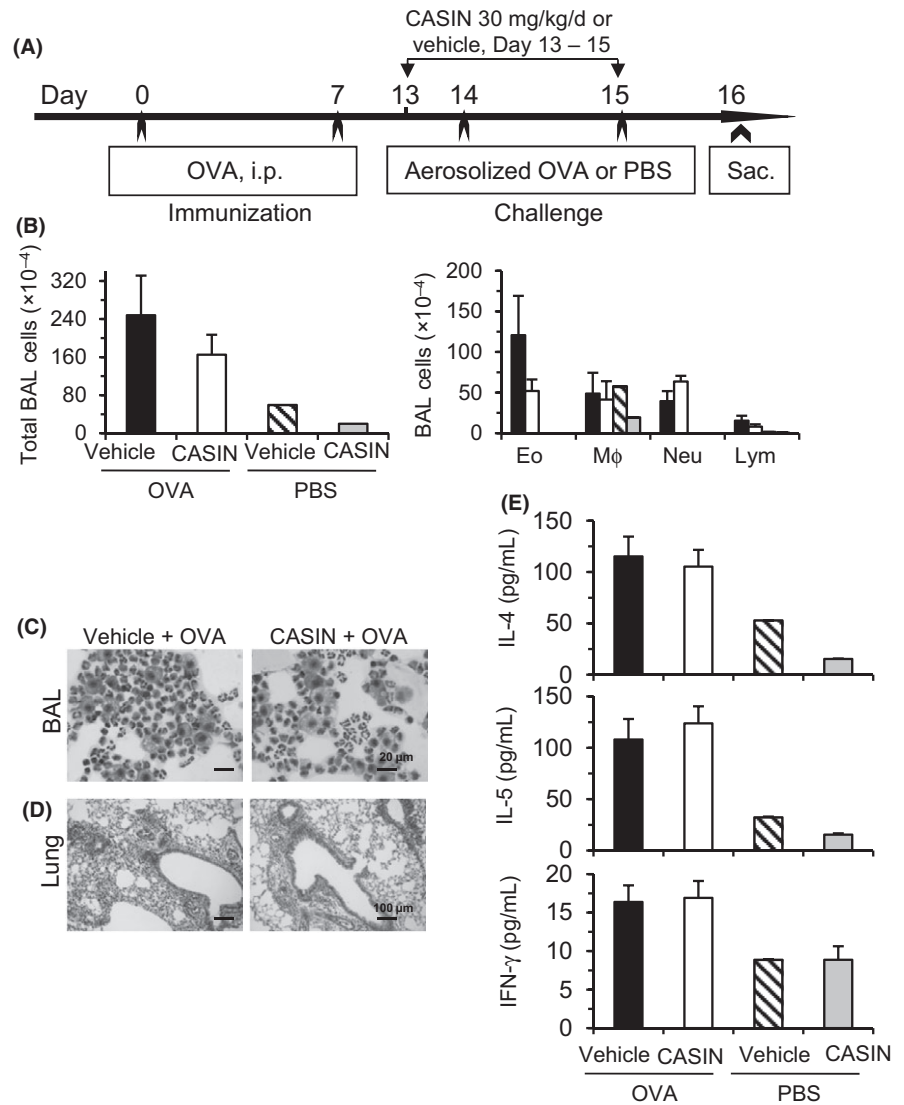


FIGURE 6 Administration of CASIN after immunization has no effect on allergic airway inflammation. A, WT mice were immunized ip with OVA on day 0 and day 7. On day 14 and 15, mice were challenged with aerosolized OVA or PBS. CASIN (30 mg/kg) or vehicle was injected ip at days 13, 14 and 15. Mice were killed 24 h after the last challenge. B-D, Total BAL cells and differential cell counts (B), representative Kwik-Diff staining for BAL cytopins (C), H&E staining of lung tissue sections (D) and cytokine levels in BAL fluids (E) are shown. Error bars represent SE. $n = 6-8$

2.6 | 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 (BD Bioscience) was used for intracellular cytokine staining. BrdU incorporation was assayed by a BrdU Flow kit following the manufacturer's protocol (BD Bioscience). Apoptosis was evaluated with an Annexin-APC Flow kit (BD Bioscience) following the manufacturer's instructions. Stained cells were analysed by FACSCalibur or FACSCanto with FACSDiva (BD Bioscience) or FCS Express (De Novo Software, Los Angeles, CA, USA) software.

3 | RESULTS

3.1 | Post-thymic deletion of Cdc42 does not affect thymocyte development and CD4⁺ T cell homeostasis but dampens CD8⁺ T cell homeostasis

To investigate the effects of Cdc42 deletion on Th2 cell differentiation and function, we crossed Cdc42^{flox/flox} mice with dLCK^{iCre} mice

in which Cre expression is controlled by distal Lck promoter and begins at a late stage of thymocyte development (Figure S1A),²² in contrast to LCK^{Cre} whose expression starts at early stage of thymocyte development (CD4⁺CD8⁺ thymocytes) (Figure S1A). The resultant Cdc42^{flox/flox}dLCK^{iCre} (hereafter referred to as Cdc42^{-/-}) mice lost Cdc42 expression in peripheral T cells but not thymocytes (Figure S1B). The mutant mice showed intact thymocyte development. The frequency and/or absolute numbers of total thymocytes and all thymocyte subsets were similar between wild-type (WT: Cdc42^{flox/flox}) and Cdc42^{-/-} mice (Figure S1C). Although the frequency of CD4⁺Foxp3⁺CD25⁺ nTreg cells was modestly decreased, their absolute numbers were not changed (Figure S1D).

In the periphery, both the frequency and absolute numbers of CD8⁺ T cells were reduced in Cdc42^{-/-} mice (Figure S1E). However, Cdc42 deficiency did not affect CD4⁺ T cell homeostasis (Figure S1E). Analysis of naïve (CD62L⁺CD44⁻) vs effector memory (CD62L⁻CD44⁺)/central memory (CD62L⁺CD44⁺) T cells revealed that naïve T cells were reduced but CD44⁺ effector memory/central memory T cells were increased in Cdc42^{-/-} CD8⁺ T cell compartment, whereas these T cell subsets remained unchanged in Cdc42^{-/-}

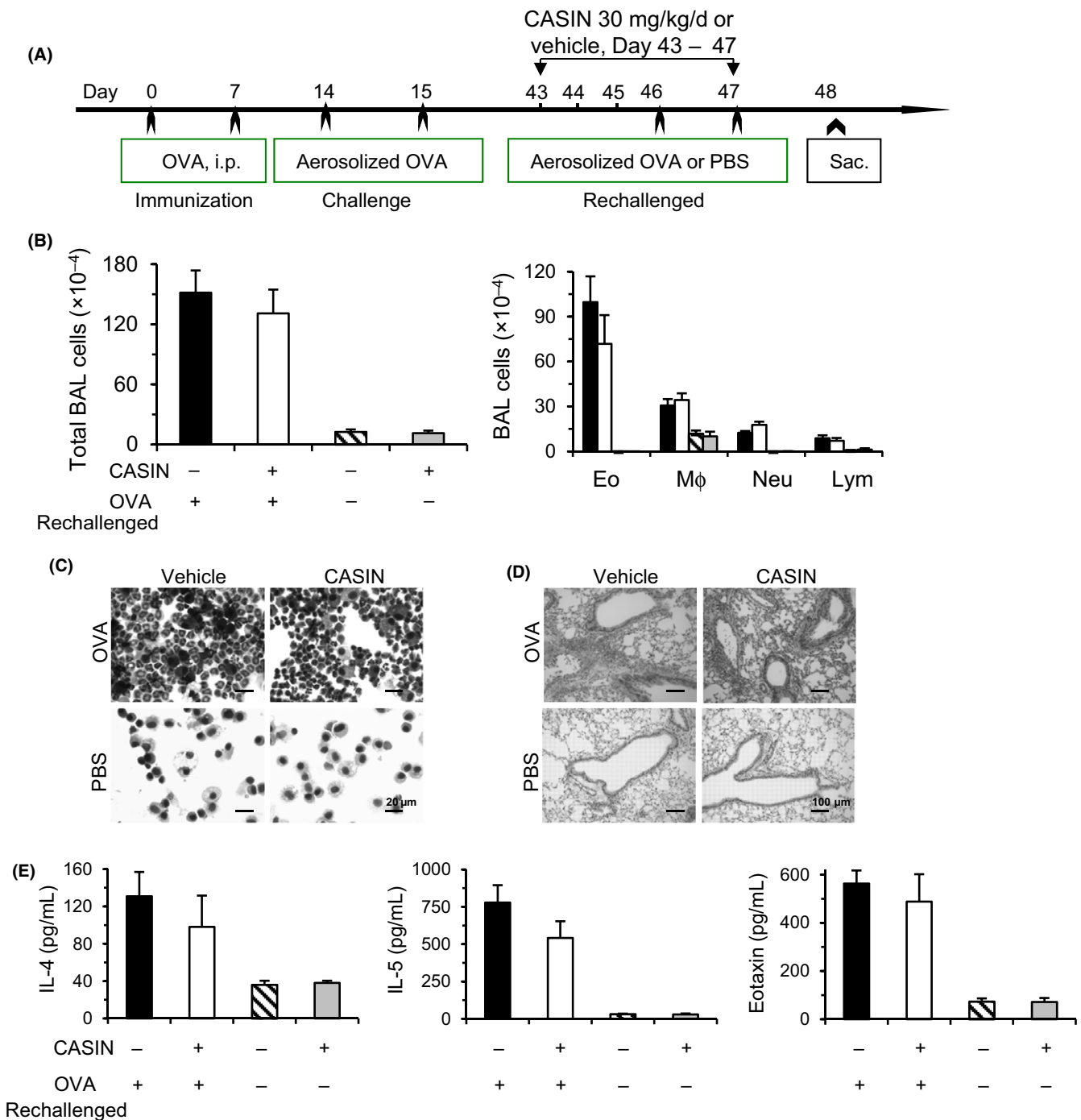


FIGURE 7 CASIN does not inhibit recurring allergic airway inflammation. WT mice were immunized ip with OVA and then challenged with aerosolized OVA for 2 days to establish allergic airway inflammation. After resting 28 days, the mice were injected ip with CASIN or vehicle for 5 days and re-challenged with aerosolized OVA or PBS for 2 days starting 3 days after the first CASIN or vehicle treatment (A). Total BAL cells and differential cell counts (B), representative Kwik-Diff staining for BAL cytopins (C), H&E staining of lung tissue sections (D), cytokine and eotaxin levels in BAL fluids (E) are shown. Error bars represent SE. $n = 4-6$ [Colour figure can be viewed at wileyonlinelibrary.com]

CD4⁺ T cell compartment (Figure S1F). Consistent with these results, in vivo BrdU incorporation assay found that CD8⁺ but not CD4⁺ T cells in Cdc42^{-/-} mice underwent homeostatic proliferation (Figure S2A). In addition, Annexin V/7AAD staining revealed an increased apoptosis in Cdc42^{-/-} CD8⁺ but not CD4⁺ T cells (Figure S2B). The lack of effect of Cdc42 deletion on CD4⁺ T cell

homeostasis might be due to less efficient deletion of Cdc42 by dLCK^{iCre} whose recombination is known to be less frequent in CD4⁺ T cells than in CD8⁺ T cells.²² We thus performed genotyping of Cdc42 in splenic CD4⁺ and CD8⁺ T cells from WT and Cdc42^{-/-} mice. We found that Cdc42 gene was effectively deleted in both CD4⁺ and CD8⁺ T cells from Cdc42^{-/-} mice (Figure S3). Collectively,

these data suggest that Cdc42 regulates CD8⁺ but not CD4⁺ T cell homeostasis.

3.2 | Post-thymic Cdc42 deficiency inhibits T cell activation in CD4⁺ but not CD8⁺ T cells and clonal expansion in both CD4⁺ and CD8⁺ T cell compartment

We next evaluated the impact of Cdc42 deficiency on T cell activation and clonal expansion. We found that anti-CD3/CD28-stimulated T cell activation (Figure S4A), activation-induced cell death (Figure S4C) and clonal expansion (Figure S5A) were all significantly downregulated in Cdc42^{-/-} CD4⁺ T cells. However, only clonal expansion was impaired in Cdc42^{-/-} CD8⁺ T cells (Figure S5B vs Figure S4B and Figure S4D). Therefore, in CD4⁺ T cells, Cdc42 appears to regulate both cell activation and clonal expansion, whereas in CD8⁺ cells, Cdc42 regulates clonal expansion but not activation.

3.3 | Post-thymic Cdc42 deficiency selectively suppresses Th2 cell differentiation

We then determined whether Cdc42 deletion affected Th and iTreg cell differentiation. Cdc42-deficient T cells appeared to retain their ability to differentiate into Th1 cells. Thus, the frequency of IFN- γ -producing cells and IFN- γ secretion were similar between Cdc42-deficient and WT T cells under polarized differentiation conditions (Figure 1A,B). The expression of T-Bet, a key Th1 transcriptional factor, was also not altered by the loss of Cdc42 (Figure 1C). Cdc42 deficiency also had no effect on TGF- β /IL-6-induced Th17 differentiation. As such, the frequency of IL-17⁺ cells, IL-17 secretion and Th17 transcription factor ROR γ t were not affected by the loss of Cdc42, under Th17-skewing condition (Figure 1D-F). Furthermore, Cdc42 deficiency did not perturb TGF- β -induced differentiation of iTreg cells, as evidenced by intact Foxp3⁺ cells (Figure 1G). In contrast to its lack of effect on Th1, Th17 and iTreg cells, deficiency of Cdc42 significantly attenuated Th2 cell differentiation. In support, IL-4-producing cells, IL-4 secretion and the activation/expression of Stat6 and GATA3, two transcriptional factors important for Th2 cell differentiation, were dramatically reduced in Cdc42-deficient T cells under neutral and/or polarized differentiation conditions (Figure 2A-D). Taken together, these data indicate that Cdc42 selectively regulates Th2 cell differentiation.

To explore the molecular mechanisms underlying Cdc42-regulated Th2 differentiation, we profiled global gene expression changes in Cdc42^{-/-} Th2 cells by RNA-Seq. Ingenuity pathway analysis (IPA) of the differentially expressed genes ($P < 0.05$) revealed that Cdc42

deficiency affected a number of pathways such as T helper cell differentiation pathway, 4-1BB signalling in T lymphocytes and signalling that regulates nucleic acid metabolism (Figure S6A,B). Quantitative real-time RT-PCR verified the upregulation of TRAF1 and TNFRSF9 in 4-1BB pathway and of CMPK2 and CMAS in nucleic acid metabolism in Cdc42^{-/-} Th2 cells (Figure S6C). IFN- γ in Th2 culture supernatants (Figure S6D) and Foxp3 expression (Figure S6E) were similar between both genotypes. These results suggest that Cdc42 plays an essential role in the regulation of immune signalling and nucleic acid metabolism in Th2 cells.

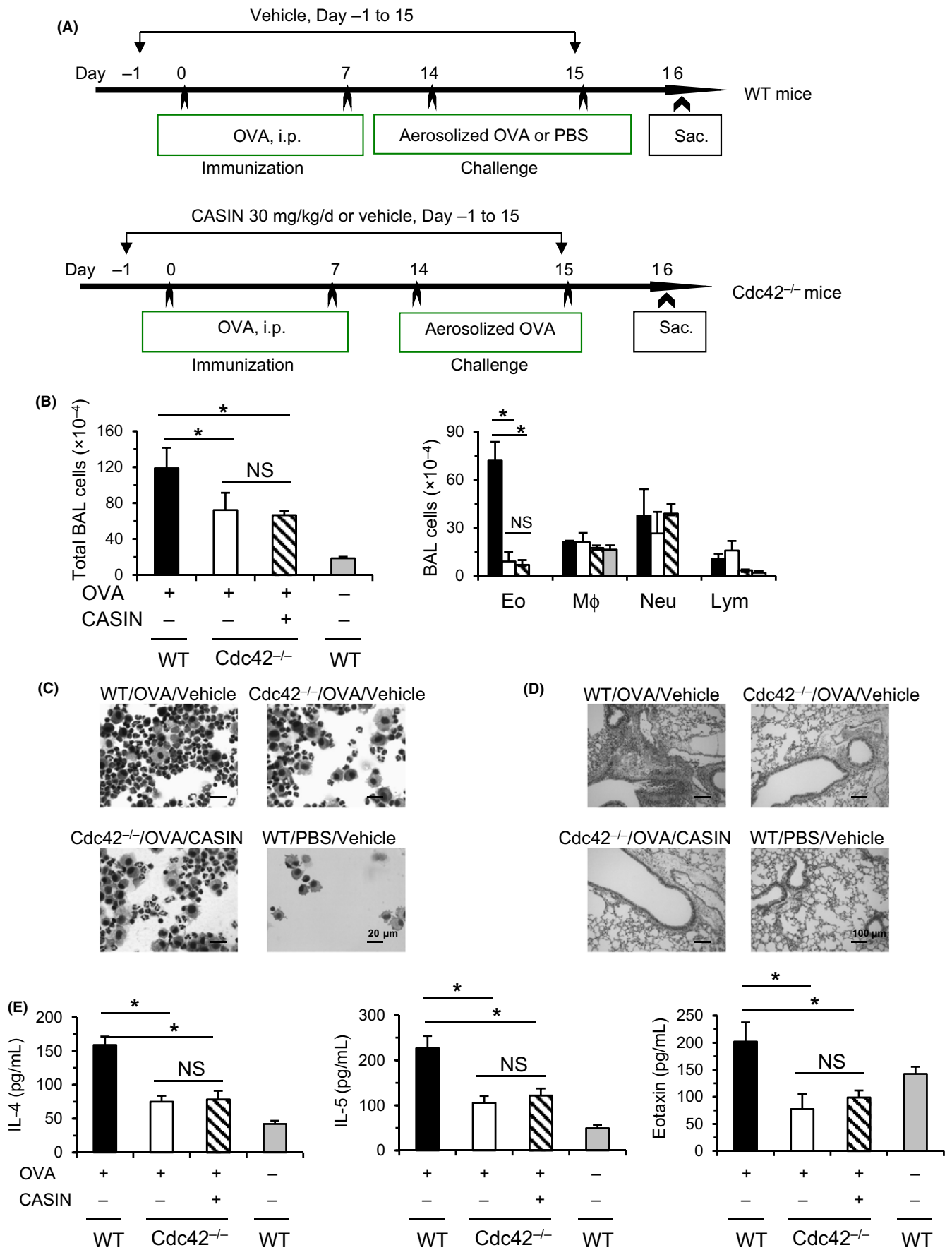
3.4 | Post-thymic Cdc42 deficiency ameliorates OVA-induced allergic airway inflammation

Since Cdc42 regulates Th2 differentiation, we investigated whether Cdc42 was necessary for an optimal Th2-mediated inflammatory response in vivo in a classical OVA-induced mouse model of asthma (Figure 3A).²³⁻²⁵ Total numbers of BAL cells in WT mice challenged with OVA had over 100-fold increase as compared to PBS-challenged mice (Figure 3B). Differential cell counting revealed that eosinophils accounted for most of this increase (Figure 3B,C). However, Cdc42 deficiency led to a dramatic reduction in total numbers of BAL cells, particularly owing to markedly decreased eosinophils (Figure 3B,C). Lung histological analysis found that OVA-challenged WT mice showed a prominent inflammatory response with massive perivascular and peribronchial infiltration, whereas this inflammatory response was markedly reduced in Cdc42-deficient mice (Figure 3D).

We reasoned that the inhibitory effect of Cdc42 deficiency on allergic airway inflammation might be attributed to reduced Th2 cytokine secretion in Cdc42^{-/-} mice as seen in vitro. In support of this notion, IL-4 and IL-5 in BAL fluids from Cdc42^{-/-} mice were markedly reduced in response to OVA challenge, whereas Th1 cytokine IFN- γ and Th17 cytokine IL-17 were not affected by the loss of Cdc42 (Figure 3E). The mRNA levels of these cytokines in the lungs followed similar patterns as protein levels in BAL (Figure 3F). Eotaxin and MUC-5AC, two non-T cell-derived mediators of allergic airway inflammation,^{24,31,32} were also downregulated by Cdc42 deficiency (Figure 3E,F). Furthermore, serum OVA-specific IgE was decreased in Cdc42^{-/-} mice (Figure 3G).

The results from the asthma model are consistent with a crucial role of Cdc42 in Th2 cell differentiation. To show direct evidence that Cdc42 modulates Th2 differentiation in vivo, we isolated trachea lymph node cells from OVA-challenged WT and Cdc42^{-/-} mice, cultured the cells in the presence anti-CD3/CD28 for 2 days and then detected Th2 cytokines in the culture supernatants. We found that secretion of IL-4 and IL-5 from Cdc42^{-/-} cells was significantly reduced compared to that from WT cells (Figure 3H). Together,

FIGURE 8 CASIN in vivo treatment does not have off-target effect on allergic airway inflammation. WT and Cdc42^{-/-} mice were immunized and challenged with OVA or PBS and injected ip daily with CASIN or vehicle (A). Total BAL cells and differential cell counts (B), representative Kwik-Diff staining for BAL cytopins (C), H&E staining of lung tissue sections (D), Th2 cytokine and eotaxin levels in BAL fluids (E) are shown. Error bars represent SE. $n = 3-5$. * $P < 0.05$. NS: no significance [Colour figure can be viewed at wileyonlinelibrary.com]



these data support that Cdc42 regulates allergic airway inflammation through its effect on Th2 cell differentiation.

3.5 | Pharmacological targeting Cdc42 suppresses Th2 differentiation and allergic airway inflammation

The inhibitory effects of genetic targeting Cdc42 on Th2 differentiation and allergy airway inflammation suggest that Cdc42 is a biologic target for asthma treatment. To test this notion, we investigated the effects of pharmacological targeting Cdc42 by CASIN, a novel Cdc42 activity-specific inhibitor developed by us.²⁰ CASIN (10 μ mol/L) inhibited anti-CD3/CD28-induced activation of Cdc42 and its immediate downstream effector molecules PAK1 and PAK2 in T cells (Figure S7A). CASIN (30 mg/kg body weight)-treated mice showed normal thymocyte development (Figure S7B). In vivo CASIN treatment also had no effect on peripheral CD4⁺ and CD8⁺ T cell homeostasis (Figure S7C). However, 10 μ mol/L and 20 μ mol/L of CASIN inhibited CD4⁺ T cell activation in vitro in a dose-dependent manner (Figure S8). Although it decreased IFN- γ ⁺ Th0 cells, 10 μ mol/L of CASIN had no effect on Th1, Th17 and iTreg cell differentiation (Figure 4A-C and Figure S9A-C), reminiscent of post-thymic deletion of Cdc42. Importantly, 10 μ mol/L of CASIN recapitulated post-thymic deletion of Cdc42 in inhibition of Th2 cell differentiation (Figure 4D and E and Figure S9D); 20 μ mol/L of CASIN suppressed all of Th1, Th2, Th17 and iTreg differentiation (data not shown), indicative of its toxicity and/or off-target effect.

Next, we examined the effect of CASIN on allergic airway inflammation. WT mice were treated with CASIN daily starting one day before OVA immunization until one day before killing (Figure 5A). We found that CASIN treatment significantly inhibited allergic airway inflammation. The infiltrating cells, particularly eosinophils, in BAL fluids (Figure 5B and C) and the lungs (Figure 5D) were markedly reduced in CASIN-treated mice. Consistently, inflammatory mediators including Th2 cytokines IL-4 and IL-5, Eotaxin, MUC-5AC, Gob5, and IL-33 in BAL fluids and/or the lungs were downregulated, whereas Th1 cytokine IFN- γ and Th17 cytokine IL-17 were not changed by CASIN treatment (Figure 5E,F). Upon in vitro restimulation with anti-CD3/CD28, trachea lymph node cells from OVA-challenged CASIN-treated mice showed decreased secretion of Th2 cytokines IL-4 and IL-5, but not Th1 cytokine IFN- γ (Figure 5G), suggesting that CASIN suppresses allergic airway inflammation through its effect on Th2 cell differentiation. Interestingly, when CASIN treatment started after OVA immunization but

before OVA challenge (Figure 6A), there was only a slight reduction in BAL cells (Figure 6B,C). The infiltrating cells in the lungs appeared similar (Figure 6D) and Th2 cytokines in BAL fluids also did not differ significantly (Figure 6E). As OVA immunization and challenge are thought to induce Th2 differentiation and inflammatory cell infiltration, respectively, our data suggest that CASIN, when employed before OVA immunization, alleviates allergic airway inflammation by inhibiting Th2 differentiation but not inflammatory cell recruitment into the lungs. Together, these results indicate that CASIN prevents the development of allergic airway inflammation/asthma.

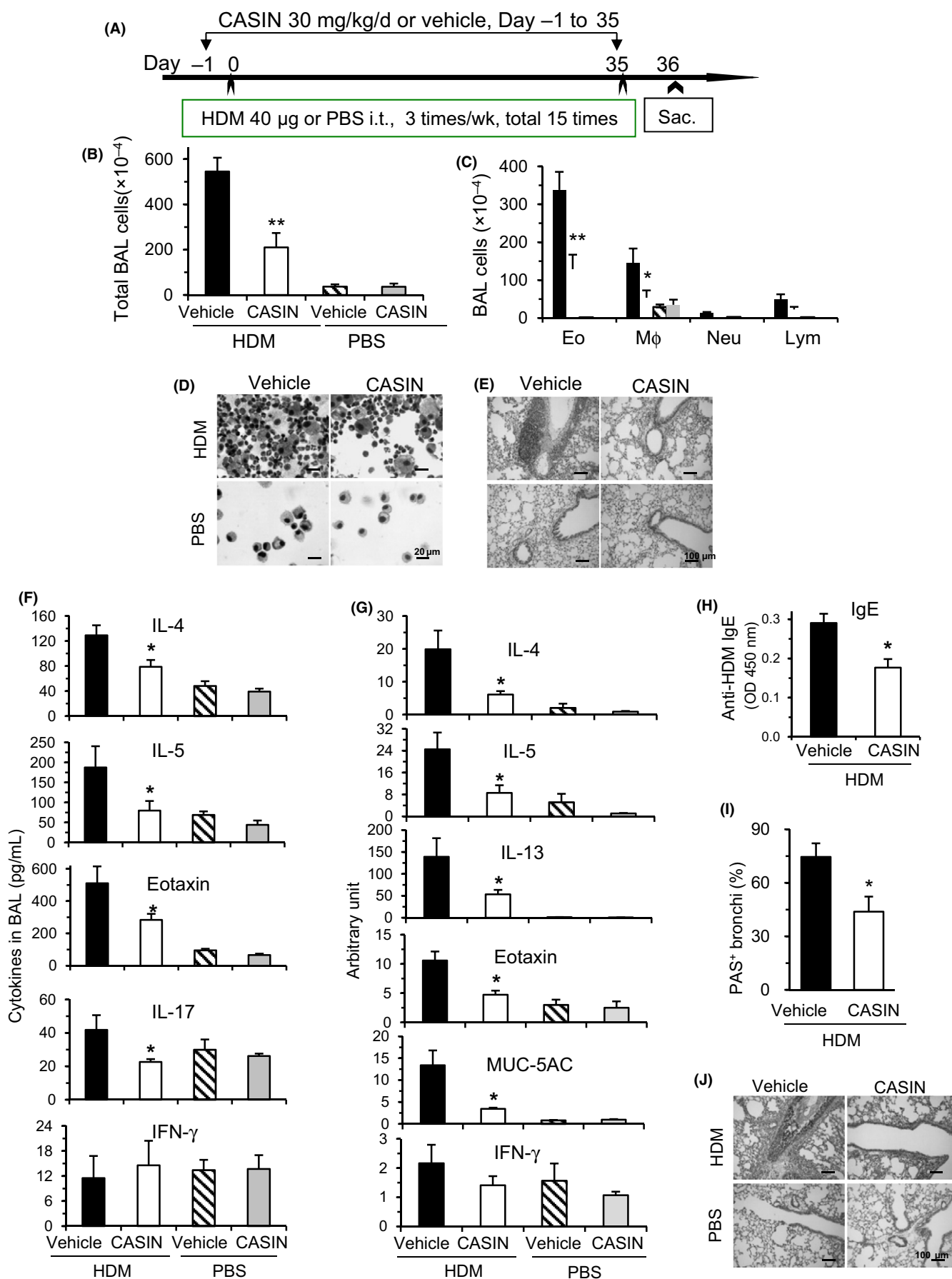
3.6 | Pharmacological targeting Cdc42 has no effect on established allergic airway inflammation

We then investigated whether CASIN could ameliorate established allergic airway inflammation. WT mice were immunized and challenged with OVA to induce allergic airway inflammation. Thirty-one days after the last OVA challenge, the mice were re-challenged with OVA and treated with CASIN amid OVA re-challenge (Figure 7A).³³ We found that CASIN- and vehicle-treated mice had comparable BAL cells (Figure 7B,C), inflammatory cell contents in the lungs (Figure 7D) and Th2 cytokines and Eotaxin in BAL fluids (Figure 7E). These data suggest that CASIN is not a therapeutic agent for allergic airway inflammation/asthma.

3.7 | The effect of pharmacological targeting Cdc42 on prevention of allergic airway inflammation development is on-target effect

Next, we examined whether the preventive effect of CASIN on allergic airway inflammation that we observed in Figure 5 was on-target effect. To this end, WT mice were immunized with OVA and challenged with OVA or PBS without CASIN treatment, whereas Cdc42^{-/-} mice were treated with CASIN or vehicle throughout OVA immunization and challenge (Figure 8A). We found that while Cdc42^{-/-} mice showed a reduction in OVA challenge-induced BAL cells (Figure 8B,C), inflammatory cell infiltration into the lungs (Figure 8D), and Th2 cytokines and Eotaxin in BAL fluids (Figure 8E), as seen in Figure 3, CASIN treatment did not cause additive inhibition of allergic airway inflammation (Figure 8B-E). Thus, CASIN prevents allergic airway inflammation development through its inhibition of Cdc42 activity in Th2 cells.

FIGURE 9 CASIN inhibits HDM-induced allergic airway inflammation. WT mice were inoculated intratracheally (i.t.) with HDM to induce chronic allergic airway inflammation (A). Control groups were inoculated i.t. with PBS alone. Mice were killed 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, E, Representative Kwik-Diff staining of BAL cytopins (D) and H&E staining of lung tissue sections (E). F, Cytokine/chemokine levels in BAL fluids determined by ELISA. G, 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. H, HDM-specific IgE levels in the serum of mice inoculated with HDM. I, J, Percentages of PAS-stained bronchi (I) and representative PAS staining of lung tissue sections (J). Error bars represent SE. n = 4-6. *P < 0.05, **P < 0.01 vs vehicle [Colour figure can be viewed at wileyonlinelibrary.com]



3.8 | Pharmacological targeting Cdc42 prevents house dust mite (HDM)-induced allergic airway inflammation

Finally, to substantiate our observation of preventive effect of pharmacological targeting Cdc42 on allergic airway inflammation in acute OVA-induced mouse model of asthma, we examined whether CASIN could prevent allergy airway inflammation, mucus production and airway resistance/airway hyperresponsiveness in chronic HDM-induced mouse model of asthma (Figure 9A). We found that upon HDM induction, CASIN treatment reduced total BAL cells (Figure 9B) and decreased eosinophils and macrophages in BAL fluids (Figure 9C and D) and the lungs (Figure 9E). In line with this, inflammatory mediators including Th2 cytokines IL-4/IL-5/IL-13, Th17 cytokine IL-17, eotaxin and MUC-5AC in BAL fluids and/or the lungs were suppressed by CASIN (Figure 9F,G). Furthermore, CASIN inhibited HDM-specific IgE (Figure 9H) and mucus production that was revealed by PAS staining (Figure 9I,J). However, CASIN did not alter airway resistance to methacholine (Figure S10). Together, these findings suggest that CASIN prevents chronic allergic airway inflammation.

4 | DISCUSSION

We have previously shown that thymic deletion of Cdc42 beginning at an early stage of thymocyte development by LCK^{Cre} impaired thymocyte development, peripheral CD4⁺ and CD8⁺ T cell homeostasis, and iTreg differentiation but enhanced CD4⁺ and CD8⁺ T cell activation and Th1/Th17 cell differentiation with no effect on Th2 cell.¹⁷⁻¹⁹ In this study, we show that post-thymic deletion of Cdc42 by dLCK^{Cre} had no effect on thymocyte development, CD4⁺ T cell homeostasis, CD8⁺ T cell activation, and Th1/Th17/iTreg differentiation, whereas it dampened CD8⁺ T cell homeostasis, CD4⁺ T cell activation and Th2 cell differentiation. Because post-thymic deletion of Cdc42 had no effect on thymocyte development and CD4⁺ T cell homeostasis, ruling out confounding effects of thymic output on T cell homeostasis and of CD4⁺ T cell homeostasis on CD4⁺ T cell activation, the defects in CD8⁺ T cell homeostasis and CD4⁺ T cell activation suggest that Cdc42 has an intrinsic role in maintaining CD8⁺ T cell homeostasis and in promoting CD4⁺ T cell activation. Furthermore, although post-thymic Cdc42 deletion inhibited CD4⁺ T cell activation, it had no effect on Th1/Th17/iTreg differentiation. The defect in Th2 cell differentiation thus suggests that Cdc42 promotes Th2 differentiation in a Th2 cell-autonomous manner. In this context, the loss of peripheral CD4⁺ T cells upon thymic deletion of Cdc42 might be attributed to the impaired thymocyte development. The enhanced CD4⁺ and CD8⁺ T cell activation might reflect lymphopenia-induced homeostatic activation. However, we do not think that the enhanced Th1/Th17 and weakened iTreg cell differentiation are attributed to the enhanced CD4⁺ T cell activation, because thymic deletion of Cdc42 had no effect on Th2 cell differentiation. Thus, it is likely that Cdc42 prevents Th1/Th17 and promotes iTreg

differentiation in a cell-autonomous manner. Therefore, both Cdc42^{flox/flox}dLCK^{iCre} and Cdc42^{flox/flox}LCK^{Cre} mice are valuable for assessing the intrinsic role of Cdc42 in different facets of T cell biology.

Our transcriptome analysis found that Cdc42-deficient Th2 cells had increased mRNA expression of STAT4, indicative of Th1 reprogramming,¹ and of Foxp3, indicative of Treg reprogramming.^{3,11} However, we did not detect alterations in protein expression of Th1 cytokine IFN- γ and Foxp3 in Cdc42-deficient Th2 cells (Figure S6D and E), suggesting that the increased mRNA expression of STAT4 and Foxp3 is compensatory effects of the impairment of Th2 differentiation programme that is known to negatively regulate differentiation programme of other T cell lineages.¹ Moreover, we found that Cdc42-deficient Th2 had enhanced 4-1BB signalling and nucleic acid metabolism signalling, suggesting that suppression of the two signalling pathways is important for Cdc42 to promote Th2 cell differentiation. Particularly, as study of genetic deletion of TRAF1 of 4-1BB signalling pathway has suggested that TRAF1 negatively regulates Th2 cell differentiation,³⁴ it is likely that the increased TRAF1 in Cdc42-deficient Th2 cells contributes to the impaired differentiation and thus Cdc42 promotes Th2 differentiation through repression of TRAF1 expression.

Consistent with its inhibitory effect on Th2 cell differentiation, post-thymic Cdc42 deletion alleviated allergic airway inflammation. These findings suggest that pharmacological targeting Cdc42 could be beneficial for asthma treatment. However, we found that while Cdc42 inhibitor CASIN suppressed the development of allergic airway inflammation, it had no effect on established allergic airway inflammation. Thus, it appears that pharmacological targeting Cdc42 may serve as a preventive but not therapeutic approach for asthma control. Given that there are no means to prevent asthma development, CASIN may have important clinical implications in asthma prevention. This is strongly supported by the preventive effect of CASIN on chronic allergic airway inflammation induced by human allergen HDM. To our surprise, CASIN did not affect HDM-induced airway hyperresponsiveness. We reason that inhibition of Cdc42 by CASIN may have led to a compensatory activation of RhoA signalling of Rho family GTPases that is known to regulate airway resistance.³⁵⁻³⁷

Furthermore, given that 10 μ mol/L of CASIN only affected Th2 cell differentiation without affecting Th1, Th17 and iTreg cells in vitro, 30 mg/kg of CASIN did not affect thymocyte development and peripheral T cell homeostasis in mice, and that 30 mg/kg of CASIN did not inhibit Th1/Th17 cell differentiation in vivo and inflammatory cell infiltration into the lungs and cause off-target effect in its inhibition of allergic airway inflammation, pharmacological targeting Cdc42 is likely a viable approach in preventing asthma development. However, unlike post-thymic deletion of Cdc42 that selectively affects Th2 cells in allergic airway inflammation, CASIN could potentially affect other inflammatory cells such as eosinophils and mast cells as well as non-inflammatory, asthma-mediating lung epithelial cells and smooth muscle cells. In addition, CASIN may affect cells that are not involved in asthma pathobiology. Thus,

detailed toxicity/pharmacokinetic studies of CASIN are warranted in our future studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

J. Q. Y. designed and performed research, analysed data and wrote the manuscript; K. W. K. performed research and analysed data; Y.L. performed research and analysed data; X. D. performed research; P.N. performed research; G.K.K.H. designed research, analysed data and contributed vital new reagents or analytical tools; J.K. performed research and analysed data; B.R. performed research and analysed data; L.Z. analysed data; N.S. analysed data; M.R. performed research; T.W. designed and performed research, analysed data and wrote the manuscript; M.E.R. designed research, contributed vital new reagents or analytical tools, and wrote the paper; Y. Z. designed research and contributed vital new reagents or analytical tools; and F. G. designed research, analysed data, contributed vital new reagents or analytical tools, and wrote the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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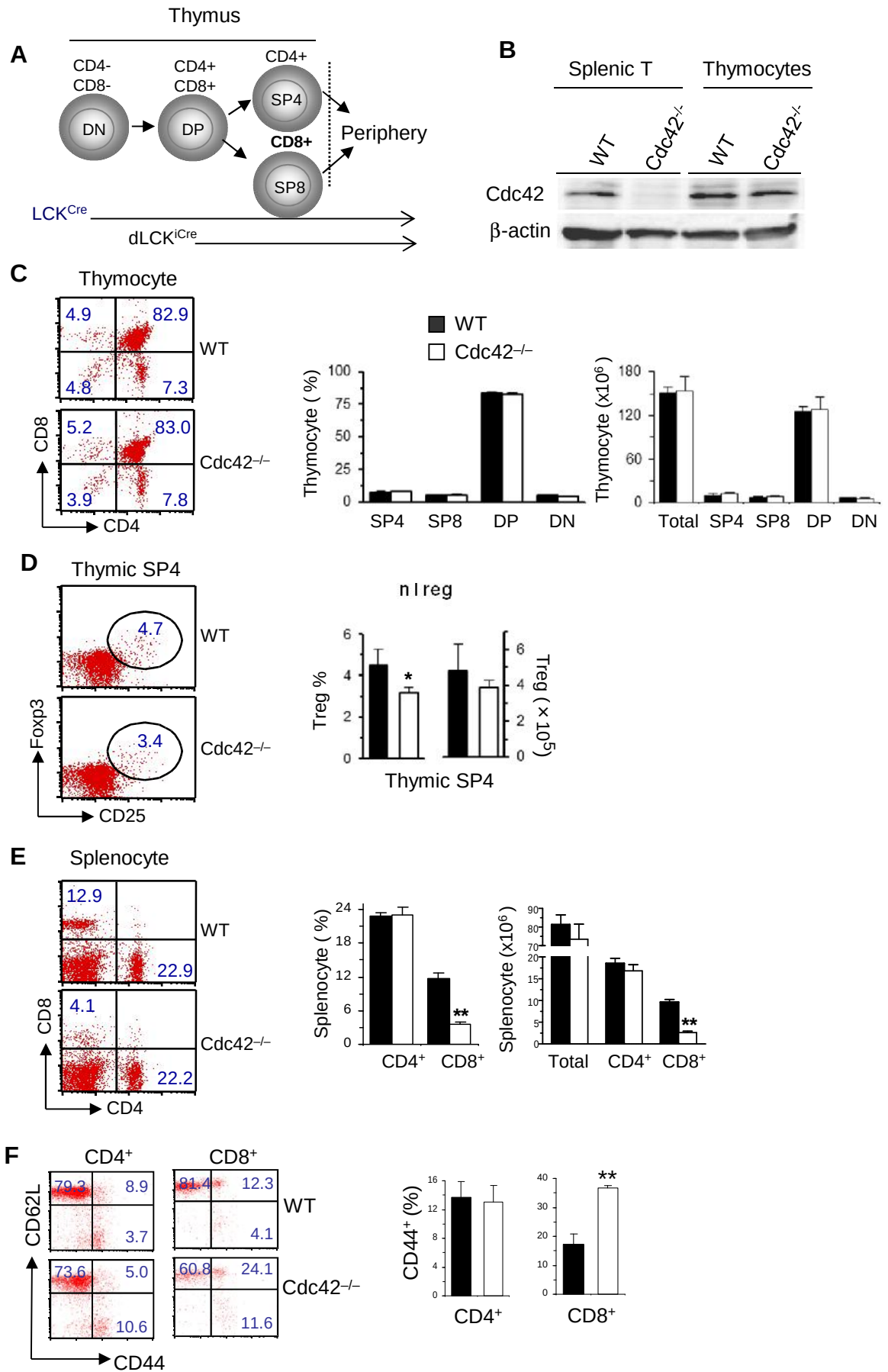


FIG S1

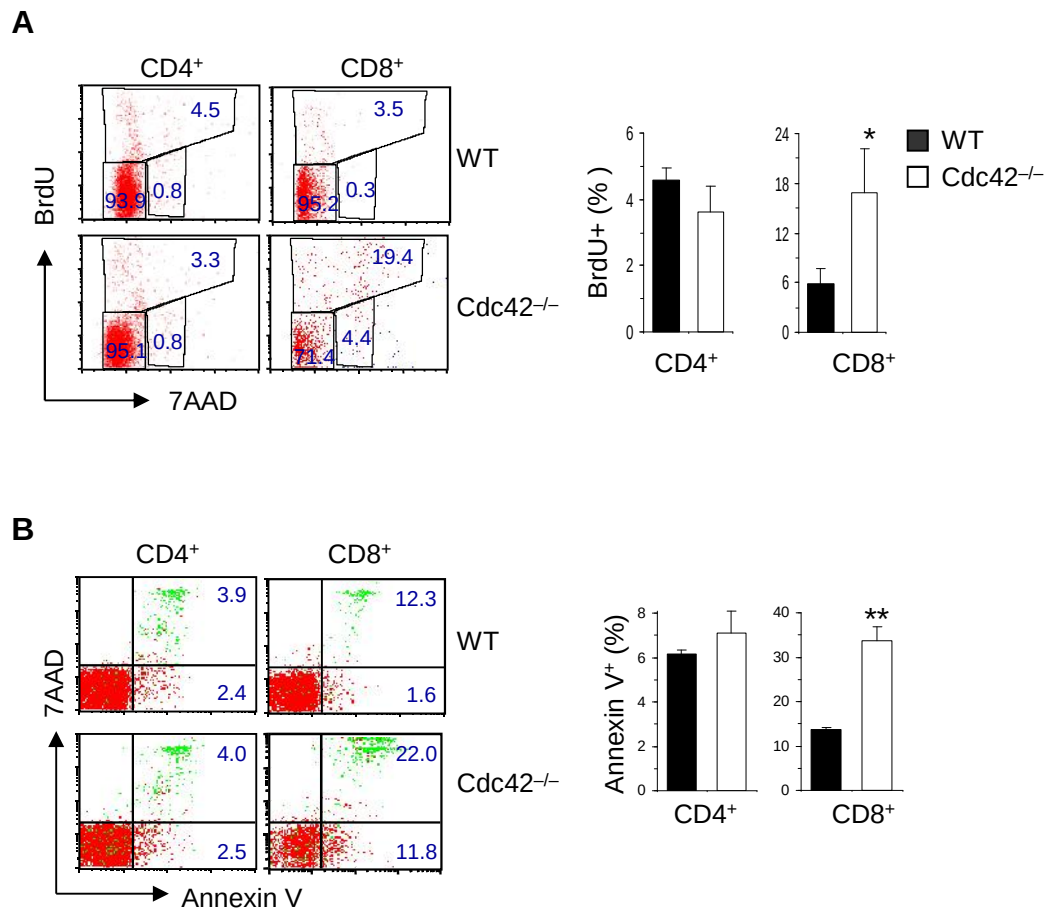


FIG S2

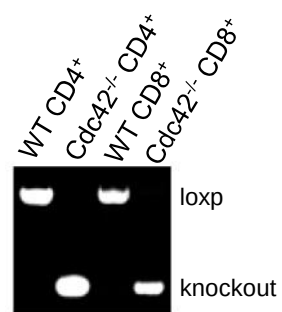


FIG S3

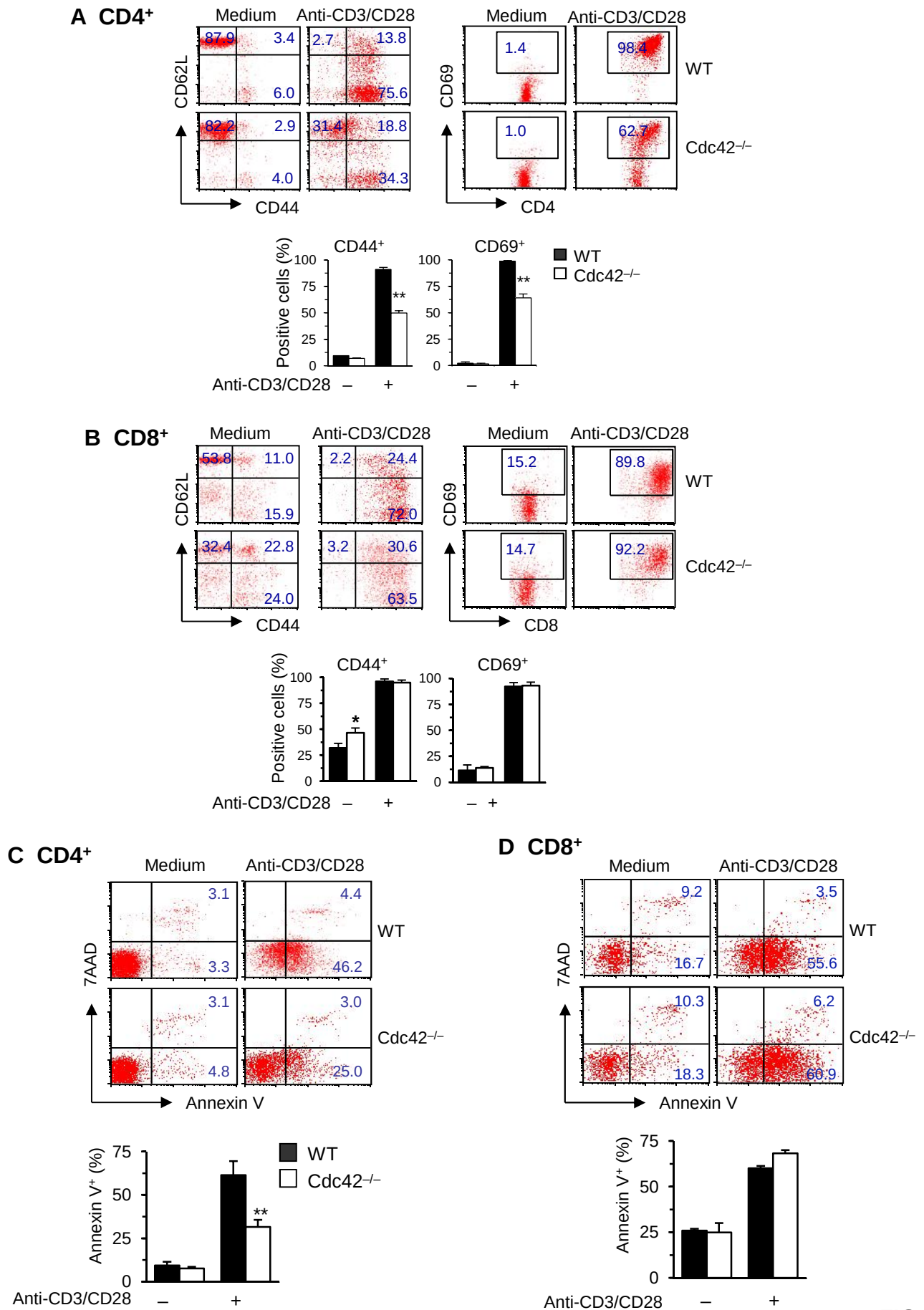
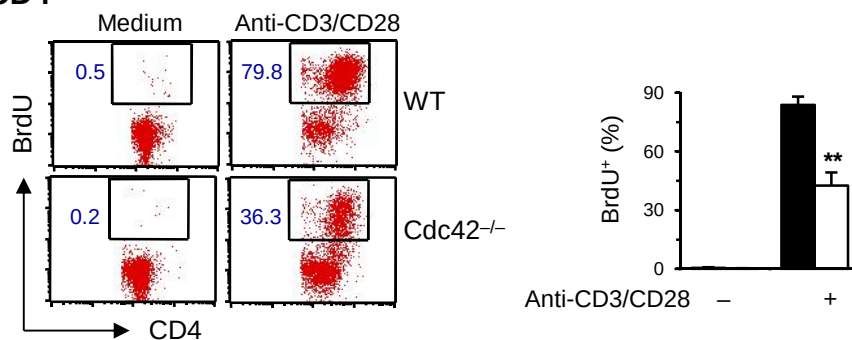


FIG S4

A CD4⁺



B CD8⁺

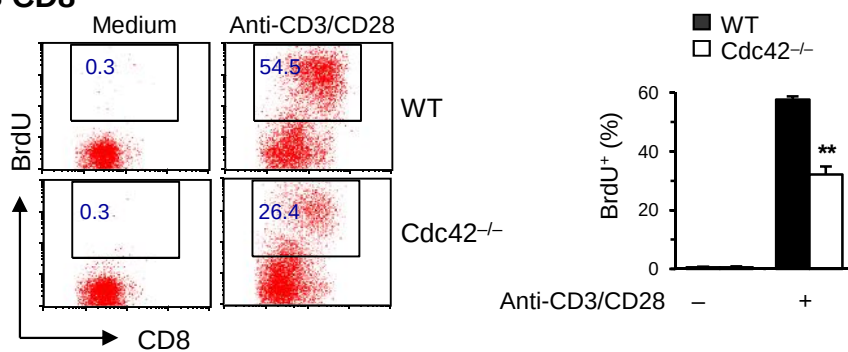
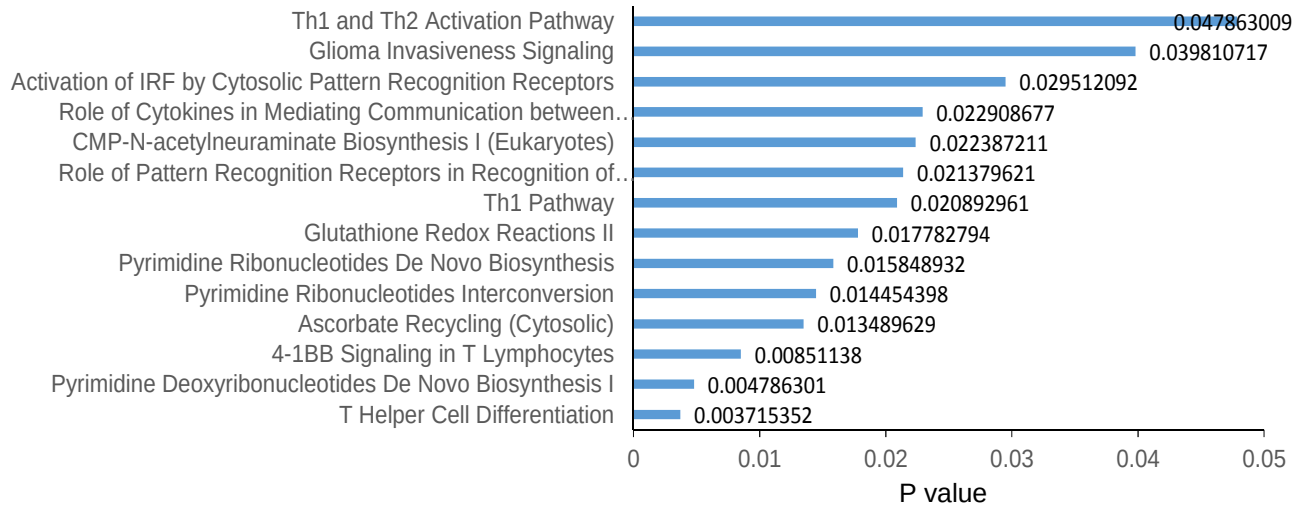
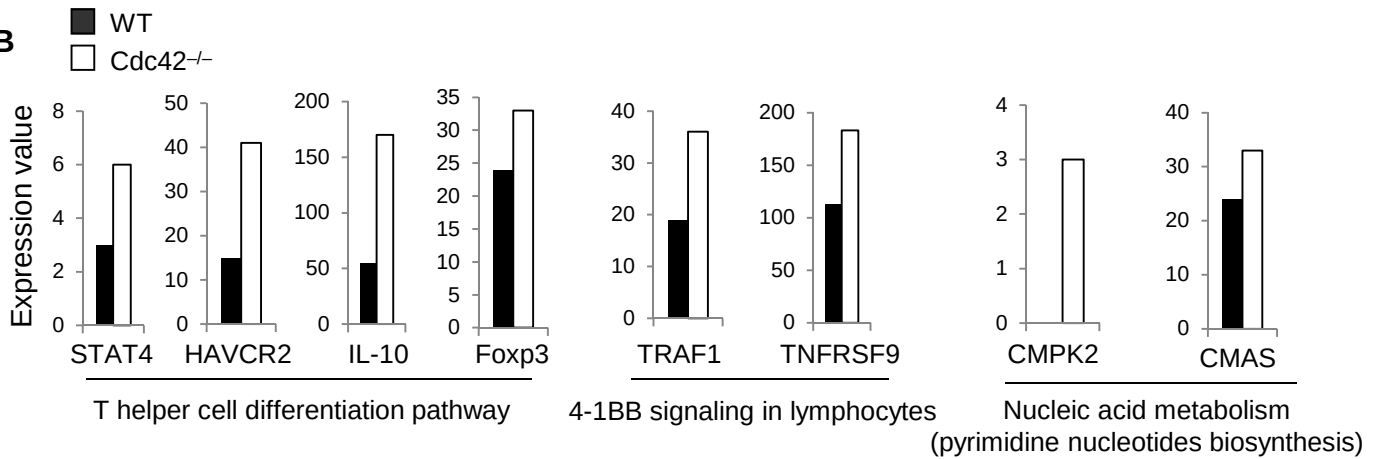
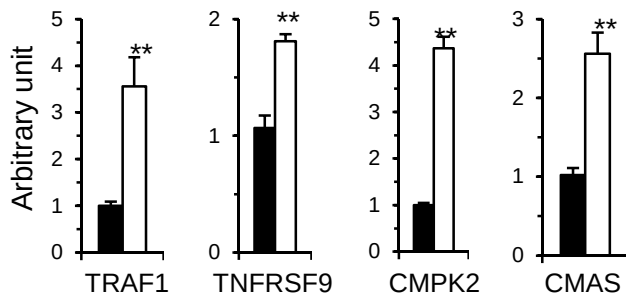
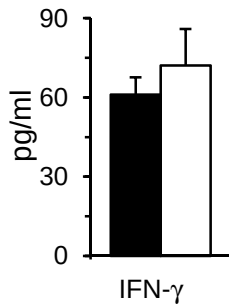
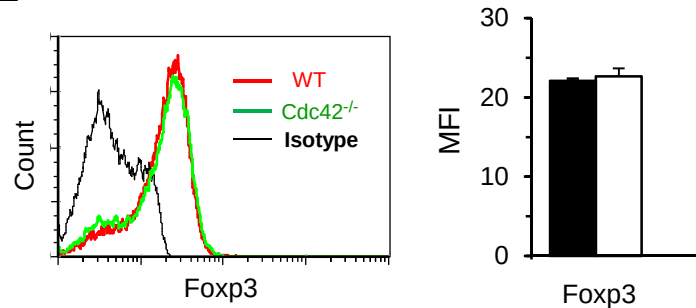


FIG S5

A**B****C****D****E****FIG S6**

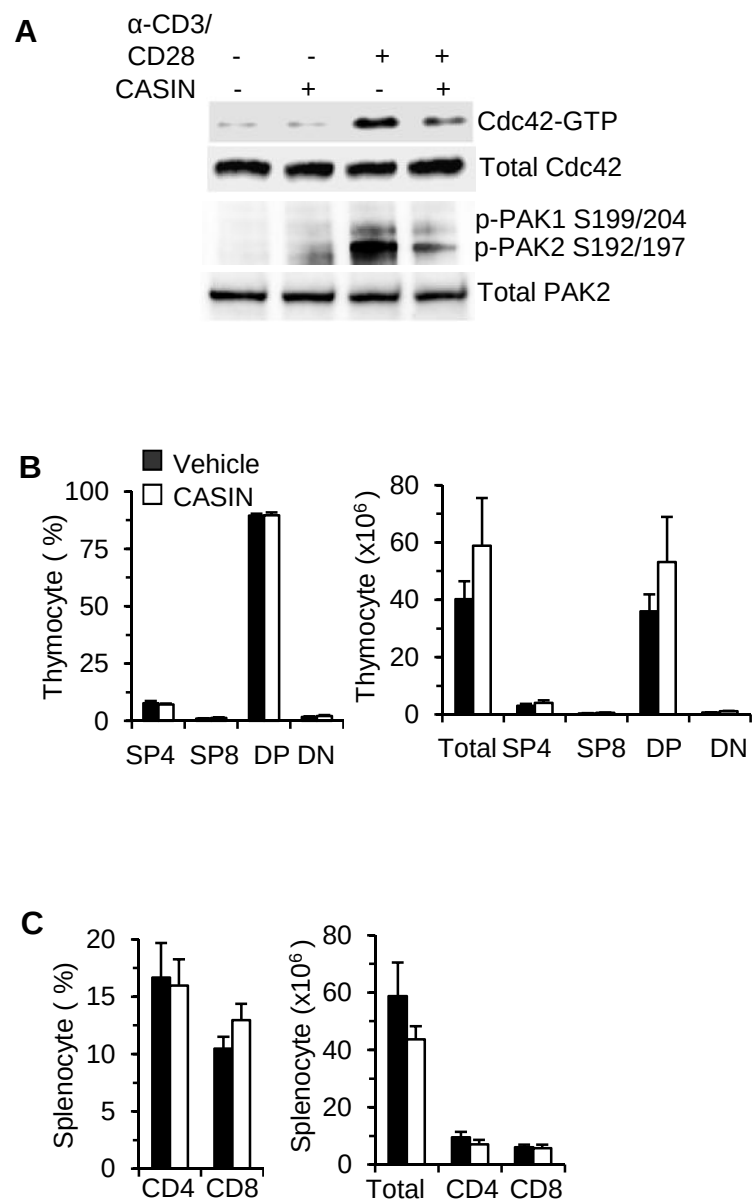


FIG S7

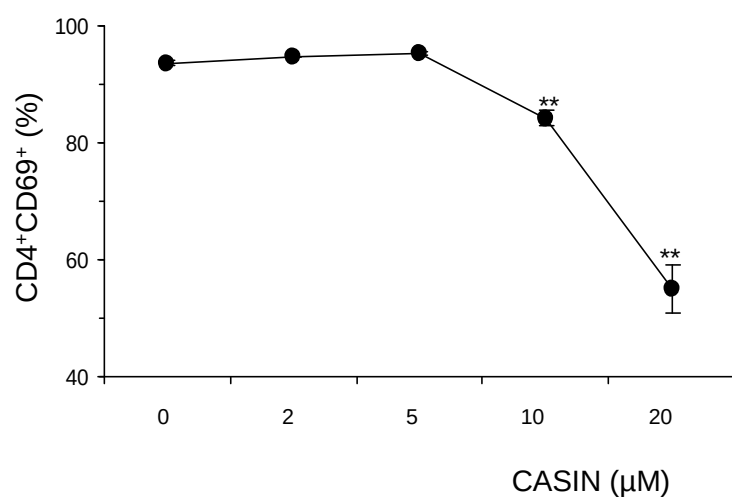
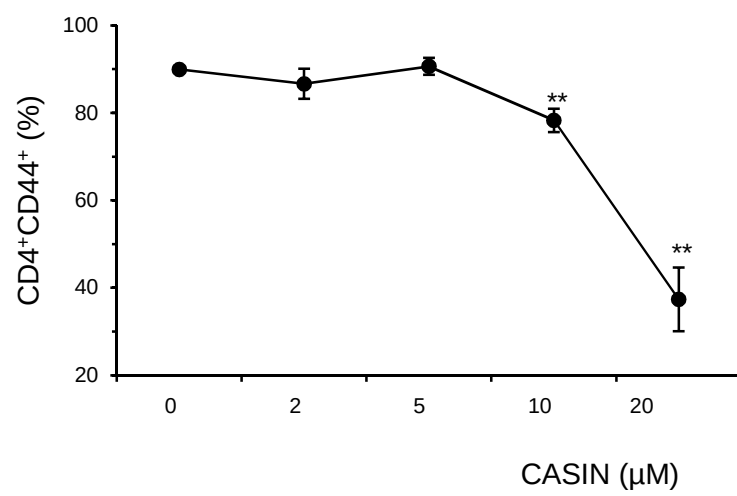


FIG S8

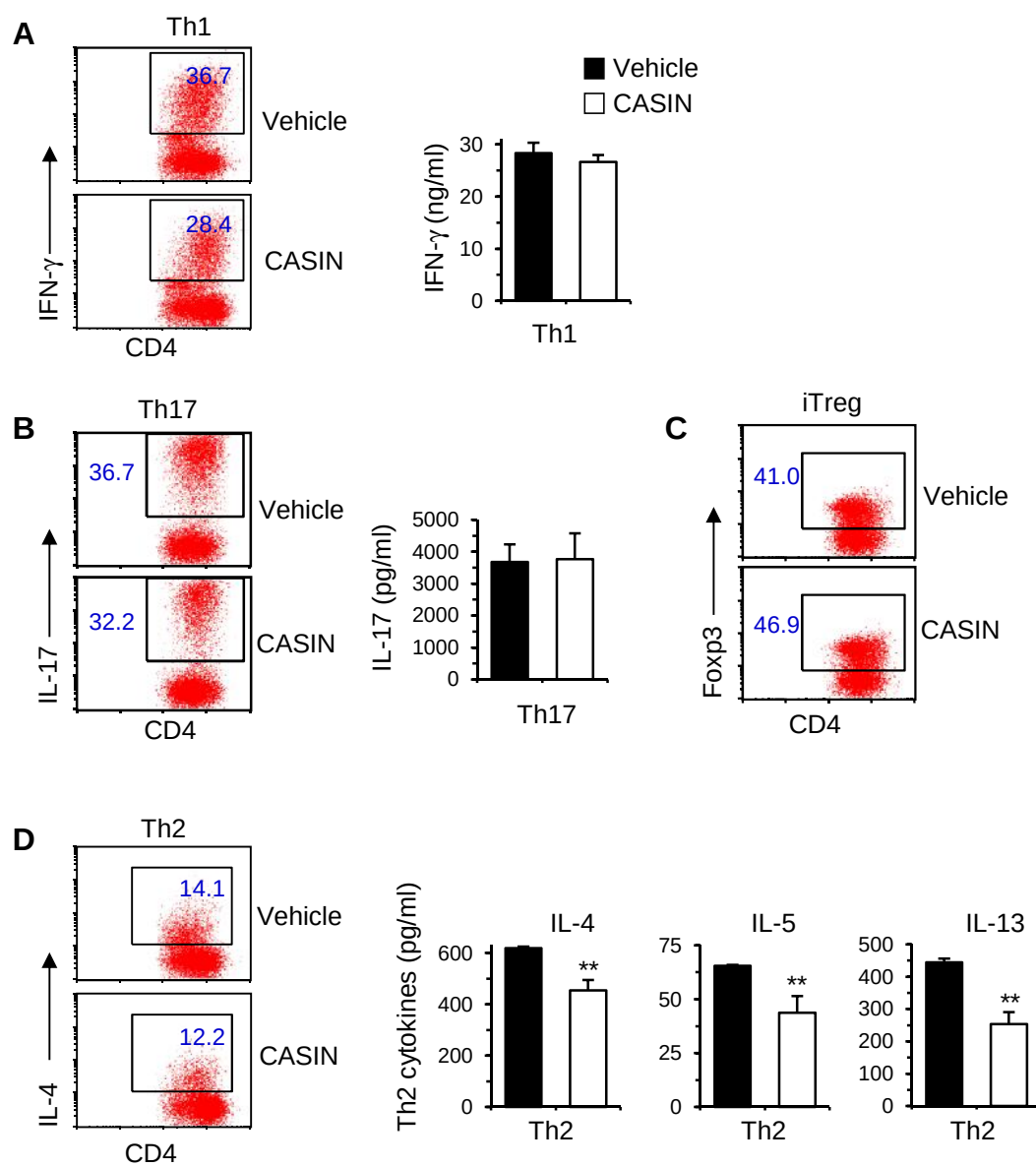


FIG S9

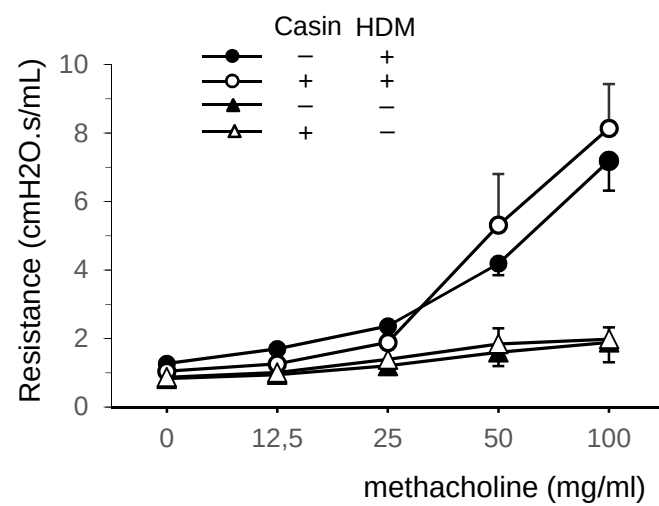


FIG S10

SUPPORTING INFORMATION

Rational targeting Cdc42 restrains Th2 cell differentiation and prevents allergic airway inflammation

(Short title: Cdc42 in Th2 cell-mediated airway inflammation)

Jun-Qi Yang, Khalid W. Kalim, Yuan Li, Xin Duan, Phuong Nguyen, Gurjit K. Khurana
Hershey, John Kroner, Brandy Ruff, Li Zhang, Nathan Salomonis, Mark Rochman, Ting Wen,
Yi Zheng, and Fukun Guo

METHODS

Western Blot

Cells were extracted with RIPA lysis buffer (1× 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 Cdc42 (67B9) (Cell Signaling, Danvers, MA), β -actin (AC-15) (Sigma), phospho-PAK1(Ser199/204)/PAK2 (Ser192/197) (Cell Signaling, Danvers, MA), or PAK2 (Cell Signaling, Danvers, MA). The bands were visualized with the enhanced chemiluminescence system (Thermo Fisher Scientific).

RNA-seq

RNA was isolated from Th2 cells with the RNeasy Mini Kit (Qiagen). The 75bp pair-end RNA-seq was performed by the Genomics, Epigenomics, and Sequencing Core at the University of Cincinnati College of Medicine. Sequence reads were aligned to the reference mouse genome (mm10) using the TopHat aligner. Reads aligning to each known transcript were counted and all follow up analyses were performed using AltAnalyze software. To elucidate the most common pathways affected by Cdc42 deletion, all genes dysregulated at least 1.5 fold were analyzed with QIAGEN's Ingenuity[®] Pathway Analysis (IPA[®], QIAGEN Redwood City, www.qiagen.com/ingenuity).

Endogenous Cdc42 activity assay. Purified splenic T cells were pretreated with or without CASIN for 1 hour and stimulated with or without 10 µg/ml anti-CD3 plus 2 µg/ml anti-CD28 antibodies for 15 minutes. Cdc42 activity (GTP-bound Cdc42) was detected by a previously described pull-down assay using the Cdc42 effector probe GST-PAK1 that contains the Cdc42-interactive domain.¹⁷

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-13, IL-17 and eotaxin were measured with DuoSet ELISA kits (R&D Systems, Minneapolis, MN). ELISA plates were developed with TMB substrate (BD Bioscience), and read with a microplate reader (Molecular Devices, Sunnyvale, CA). Cytokine mRNA levels were measured by quantitative real-time PCR.

Airway hyperresponsiveness. Invasive measurements of airway responsiveness were made on a flexiVent apparatus (Scireq, Montreal, Quebec, Canada) as previously reported.³⁸ Briefly, mice were anesthetized with ketamine, xylazine and acepromazine. Mouse tracheas were cannulated and ventilated at 150 breaths/ min and a 3.0 cm of water positive end-expiratory pressure. Two total lung capacity perturbations were then performed for airway recruitment before baseline measurement and subsequent methacholine challenges were performed. Dynamic resistance (R) and compliance (C) were determined by fitting the data to a single compartment model of airway mechanics, where P_{tr} is defined as $R V + \frac{1}{E} V + P_0$ and P_{tr} is defined as tracheal pressure, V is volume, E is elastance, P_0 is a constant, and C is defined as $1/E$. Measurements were made with a 1.25-second, 2.5-Hz volume-driven oscillation applied to the airways by using a computer-

controlled piston (SnapShot perturbation). PBS or methacholine was aerosolized for 15 seconds (Aeroneb ultrasonic nebulizer; Aerogen, Galway, Ireland), which was followed by 15 seconds of ventilation, and a SnapShot perturbation was performed, which was followed by 10 seconds of ventilation. Twelve SnapShot/ventilation cycle measurements were made. The procedure was repeated for 0, 12.5, 25, 50 and 100 mg/mL concentrations of methacholine. The maximum resistance and minimum compliance values with a coefficient of determination of 0.9 or greater (as determined by the flexiVent software) was used to determine the dose-response curve.

Statistical analysis. All experimental data were analyzed and compared for statistically significant differences using two-tailed Student's *t* or Mann-Whitney *U* test, and a *P* value of < 0.05 was considered significant.

FIGURE LEGENDS

Figure S1 Post-thymic Cdc42 deficiency does not affect thymocyte development and CD4⁺ T cell homeostasis but dampens CD8⁺ T cell homeostasis. (A) Diagram of the expression time frame of LCK^{cre} versus dLCK^{iCre} during thymocyte development. DN: double negative (CD4⁻CD8⁻); DP: double positive (CD4⁺CD8⁺); SP4: CD4 single positive; SP8: CD8 single positive. (B) Immunoblot of Cdc42 expression in thymocytes and splenic T cells from WT (Cdc42^{flox/flox}) and Cdc42^{-/-} (Cdc42^{flox/flox}dLCK^{iCre}) mice. (C) Flow cytometry analysis of thymocytes. Left, representative flow dot plots; middle, proportions of thymocyte subsets; right: absolute numbers of total thymocytes and thymocyte subsets. (D) Flow cytometry analysis of natural Treg (nTreg) cells gated on thymic SP4. Left, representative flow dot plots; right, proportions and absolute numbers of thymic nTreg. (E) Flow cytometry analysis of splenocytes. Left, representative flow dot plots; middle, proportions of CD4⁺ and CD8⁺ splenic T cells; right: absolute numbers of total splenocytes and CD4⁺ and CD8⁺ splenic T cells. (F) Total splenocytes were stained for CD4, CD8, CD62L and CD44. Percentages of CD62L⁺CD44⁻ (naïve), CD62L⁻CD44⁺ (effector memory), CD62L⁺CD44⁺ (central memory) cells are shown in representative flow dot plots gated on CD4⁺ or CD8⁺ cells (left). Percentages of CD44⁺ cells were shown in bar graphs (right). Error bars represent SD, n=10-15 (C, E) or n=5-10 (D, F). **P* < 0.05, ***P* < 0.01.

Figure S2 Post-thymic Cdc42 deficiency leads to homeostatic proliferation and increased apoptosis in CD8⁺ but not CD4⁺ T cell compartment. (A) T cell proliferation was evaluated by BrdU incorporation. Mice were injected with BrdU i.p. and sacrificed 18 h post injection. Total splenocytes were prepared and processed for CD4, CD8, BrdU, and 7-AAD staining. Representative flow dot plots gated on CD4⁺ or CD8⁺ cells are shown (Left). Percentages of

BrdU⁺ CD4⁺ or CD8⁺ cells are displayed in bar graphs (Right). (B) Total splenocytes were stained with CD4, CD8, Annexin V, and 7AAD. Representative flow dot plots gated on CD4⁺ or CD8⁺ cells are shown (Left). Percentages of Annexin V⁺ cells are displayed in bar graphs. Error bars represent SD. n=8. **P* < 0.05, ***P* < 0.01 vs. WT control group.

Figure S3 Cdc42 gene is efficiently deleted in both CD4⁺ and CD8⁺ T cells by dLCK^{iCre}. Cdc42 floxed and knockout alleles in purified CD4⁺ and CD8⁺ T cells were detected by PCR genotyping.

Figure S4 Post-thymic Cdc42 deficiency inhibits T cell activation and activation-induced cell death in CD4⁺ T cell compartment but not in CD8⁺ T cell compartment. Splenic CD4⁺ (A, C) and CD8⁺ (B, D) T cells were isolated and cultured with or without plate-bound anti-CD3 plus soluble anti-CD28 for 2 d. Cells were stained for indicated activation markers (A, B) or with Annexin V and 7-AAD (C, D). Upper, representative flow dot plots; lower, percentages of CD44⁺ and CD69⁺ cells (A, B) or Annexin V⁺ cells (C, D). Error bars represent SD, n = 8. **P* < 0.05, ***P* < 0.01.

Figure S5 Post-thymic Cdc42 deficiency inhibits clonal expansion in both CD4⁺ and CD8⁺ T cell compartment. Splenic CD4⁺ (A) and CD8⁺ (B) T cells were isolated and cultured with or without plate-bound anti-CD3 and soluble anti-CD28 for 2 d. BrdU was added into the cultures for the last 18 h. Cells were harvested and processed for BrdU staining. Left, representative flow dot plots; right, percentages of BrdU⁺ Cells. Error bars represent SD, n = 8. ***P* < 0.01.

Figure S6 Post-thymic Cdc42 deficiency alters Th cell differentiation programs, 4-1BB signaling and nucleic acid metabolism signaling in Th2 cells. Naïve CD4⁺ T cells from WT and Cdc42^{-/-} mice were differentiated under Th2 conditions for 4 days followed by PMA plus ionomycin restimulation for 5 h. Dead cells were removed by Ficoll-Paque. The cells were then analyzed for gene expression profiles by RNA-Seq (A, B). Canonical pathways altered in Cdc42^{-/-} Th2 cells (P values are shown) (A) and the expression changes of representative genes in T helper cell differentiation pathway, 4-1BB signaling pathway, and nucleic acid metabolic pathway (B) are shown. The expression changes of representative genes in 4-1BB signaling pathway and nucleic acid metabolic pathway were verified by quantitative real-time RT-PCR (C). IFN- γ in the culture supernatants was determined by ELISA (D). Mean fluorescence intensity (MFI) of Foxp3 was assayed by FACS (E). Error bars represent SD of 3 mice. ***P* < 0.01.

Figure S7 Pharmacological targeting Cdc42 has no effect on thymocyte development and peripheral T cell homeostasis. (A) Purified splenic T cells from WT mice were pretreated with or without CASIN (10 μ M) for 1 hour and stimulated with or without 10 μ g/ml anti-CD3 plus 2 μ g/ml anti-CD28 antibodies for 15 minutes. Cdc42 activity (GTP-bound Cdc42) was detected by a pull-down assay using the probe GST-PAK1 that contains the Cdc42-interactive domain. The amount of Cdc42-GTP binding to PAK1 was detected by Western blotting of the co-precipitates with anti-Cdc42 antibody. Total Cdc42 from total cell lysates was detected as loading control. Total cell lysates were also subjected to Western blot of phospho (p)-PAK1/PAK2 and total PAK2. (B, C) WT mice were injected i.p. daily with CASIN (30 mg/kg) or vehicle for 16 consecutive days. Thymocytes and splenocytes from the mice were analyzed by flow cytometry

for the proportions and absolute numbers of thymocyte subsets (*B*) and of splenic CD4⁺ and CD8⁺ T cells (*C*). Data are representative of two independent experiments. Error bars represent SE. *n* = 4-6. DN: double negative (CD4⁻CD8⁻); DP: double positive (CD4⁺CD8⁺); SP4: CD4 single positive; SP8: CD8 single positive.

Figure S8 Pharmacological targeting Cdc42 inhibits T cell activation in a dose-dependent manner. Splenic CD4⁺ T cells were isolated and cultured with plate-bound anti-CD3 plus soluble anti-CD28 for 2 d, in the presence or absence of the indicated concentrations of CASIN. Cells were stained for activation markers CD44 and CD69. Percentages of CD44⁺ and CD69⁺ cells are shown. Data are representative of 4 mice. Error bars represent SD of triplicates. *****P* < 0.01.**

Figure S9 CASIN added during restimulation selectively suppresses Th2 differentiation. Naïve WT CD4⁺ T cells were differentiated under Th1 (*A*), Th17 (*B*), iTreg (*C*) or Th2 (*D*) skewing conditions for 4 days without CASIN. The cells were restimulated with PMA plus ionomycin for 5 h in the absence (vehicle) or presence of CASIN (10 μM), then processed for surface staining of CD4 and intracellular staining of IFN-γ (*A*), IL-17 (*B*), Foxp3 (*C*) or IL-4 (*D*). Percentages of cytokines or Foxp3 positive cells are shown in representative dot plots gated on CD4⁺ cells. Cytokines in the culture supernatants by ELISA were shown in bar graphs. Error bars represent SD of triplicates. Results are representative of two independent experiments. *****P* < 0.01.**

Figure S10 CASIN does not affect airway resistance. WT mice were inoculated intratracheally (i.t.) with HDM. Control groups were inoculated i.t. with PBS alone. The mice were treated with

CASIN or vehicle as depicted in Fig. 9. Airway resistance of bronchi to methacholine was examined. Error bars represent SE. n = 4-6.