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Short Communication

SHORT COMMUNICATION

# RhoA and Cdc42 in T cells: Are they targetable for T cell-mediated inflammatory diseases?

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## Abstract

Many inflammatory diseases are not curable, necessitating a better understanding of their pathobiology that may help identify novel biological targets. RhoA and Cdc42 of Rho family small GTPases regulate a variety of cellular functions such as actin cytoskeletal organization, cell adhesion, migration, proliferation, and survival. Recent characterization of mouse models of conditional gene knockout of RhoA and Cdc42 has revealed their physiological and cell type-specific roles in a number of cell types. In T lymphocytes, which play an important role in the pathogenesis of most, if not all, of the inflammatory diseases, we and others have investigated the effects of T cell-specific knockout of RhoA and Cdc42 on T cell development in the thymus, peripheral T cell homeostasis, activation, and differentiation to effector and regulatory T cells, and on T cell-mediated allergic airway inflammation and colitis. Here we highlight the phenotypes resulting from RhoA and Cdc42 deletion in T cells and discuss whether pharmacological targeting of RhoA and Cdc42 is feasible in treating asthma that is driven by allergic airway inflammation and colitis.

**Key words:** RhoA; Cdc42; T cells; allergic airway inflammation; colitis

## Introduction

RhoA and Cdc42 belong to the Rho family small GTPases of the Ras superfamily. Like other Rho GTPases, RhoA and Cdc42 cycle between GTP-bound active and GDP-bound inactive states.[1,2] In their active form, RhoA and Cdc42 bind to and activate a number of immediate downstream effector molecules (e.g. ROCK of RhoA effectors, PAK1 of Cdc42 effectors).[3] Overexpression of dominant active or negative mutants of RhoA and Cdc42 has revealed a role for RhoA and Cdc42 in modulating actin cytoskeleton organization, cell adhesion, migration, proliferation, and survival.[4–11] For example, in T lymphocytes, overexpression of the dominant mutants suggests that RhoA plays a role in thymocyte adhesion, thymic egress, and T cell polarization.[12–17] Furthermore, similar to dominant negative mutant approach, inactivation of RhoA by C3 transferase in transgenic mice causes a blockade in thymocyte development.[18,19] Likewise, Cdc42 plays a role in thymocyte development, T cell actin and tubulin cytoskeleton polarization, and T cell migration.[20–23] However, these functions of RhoA and Cdc42 are perplexed by the nonspecific nature of the dominant mutants or C3 transferase that may affect other Rho GTPases.[24–28] In this context, distinct and cell type-specific functions of RhoA have been uncovered using RhoA knockout mice.

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For example, in contrast to the prevailing view that RhoA is critical for actin cytoskeleton rearrangement and cell adhesion, RhoA deficiency in primary mouse embryonic fibroblasts does not alter actin stress fiber and focal adhesion complex formation.[29] Therefore, the physiologic and selective role of RhoA and Cdc42 can only be revealed by a genetic approach.

In this brief review, we summarize recent progress in characterizing mouse models of gene knockout of RhoA and Cdc42 in T cells and discuss whether RhoA and Cdc42 are targetable in T cell-mediated inflammatory diseases.

## RhoA and Cdc42 in thymocyte development

Thymocyte development proceeds sequentially from $CD4^-CD8^-$ double-negative (DN), $CD4^+CD8^+$ double-positive (DP), to $CD4^+$ or $CD8^+$ single-positive (SP). The differentiation of DN thymocytes to DP cells is contingent on the rearrangement of T cell receptor (TCR) $\beta$ gene, namely V(D)J recombination, and $\beta$-selection, a process in which DN thymocytes successfully expressing rearranged TCR$\beta$ are rescued from cell death and allowed to proliferate and differentiate to DP cells. The differentiation of DP cells to SP cells relies on positive selection, a process in which DP thymocytes expressing TCR$\alpha\beta$ of low affinity to self-peptide/MHC complexes are instructed to mature to SP cells.[30,31]

By deletion of RhoA gene starting from DN thymocytes, we and others have found that RhoA is important for thymocyte development.[30,32] Mechanistically, RhoA promotes $\beta$-selection and positive selection. $\beta$-selection and positive selection are accompanied by thymocyte survival and proliferation. We have shown that RhoA is essential for thymocyte survival and proliferation likely through restraining reactive oxygen species (ROS) production to maintain ATP levels in thymocytes.[30] Interestingly, while we found that RhoA deficiency decreased $CD4^+$ SP thymocytes,[30] López-Posadas *et al.* found that deletion of RhoA starting from DP thymocytes increased $CD4^+$ SP thymocytes,[33] suggesting a developmental stage-specific role of RhoA in thymocyte development. By deletion of Cdc42 gene starting from DN thymocytes, we have found that Cdc42 is required for thymocyte development by promoting positive selection. Mechanistically, Cdc42 facilitates thymocyte proliferation, survival, and migration.[31] In contrast to the well-recognized role of Cdc42 in the regulation of cell adhesion,[4] Cdc42 is not important for thymocyte adhesion, indicating that the role of Cdc42 in cell adhesion is cell type-dependent.[31]

## RhoA and Cdc42 in T cell homeostasis and activation

After maturation in the thymus, $CD4^+$ or $CD8^+$ SP thymocytes migrate to the periphery, where they are maintained as resting, naive T cells. Naive T cells are maintained mainly by IL-7-mediated T cell survival.[34] Upon antigen recognition, naive T cells become activated and expanded.[35]

We and others found that RhoA deletion caused a reduction in naive T cell numbers and impaired T cell activation and expansion,[32,33,35] suggesting that RhoA is required for T cell homeostasis, activation, and proliferation. Mitochondrial metabolism is important for T cell activation.[36] We found that RhoA deficiency dampened oxidative phosphorylation and glycolysis in activated T cells,[35] suggesting that RhoA promotes T cell activation through regulating mitochondrial function. Cdc42 deficiency mimicked RhoA deficiency in reducing T cell numbers, attributable to a defect in IL-7-mediated survival signaling.[34] Interestingly, unlike RhoA deficiency that attenuated T cell activation and expansion, depletion of Cdc42 enhanced T cell activation and expansion.[34] Therefore, Cdc42 functions to maintain T cell homeostasis and limit T cell activation and proliferation. Cdc42 may do so through its immediate downstream effector PAK1.[34]

## RhoA and Cdc42 in effector and regulatory T cell differentiation

Upon activation and expansion, naive T cells differentiate to either effector T cells or induced regulatory T (iTreg) cells. $CD4^+$ naive T cells can differentiate to several types of effector T helper (Th) cells, among which Th1, Th2 and Th17 cells have been well studied. Maturation of Th1, Th2 and Th17 cells requires discrete cytokine signals. Th1 cell differentiation requires IFN-$\gamma$-elicited STAT1 and IL-12-triggered STAT4 signaling cascades, Th2 cell differentiation requires IL-4-mediated STAT6 signaling pathway, and Th17 cell differentiation requires IL-6-mediated STAT3.[1–3] The cytokine signaling in Th1 cells promotes expression and/or DNA binding of transcription factor T-bet, leading to the synthesis of Th1 signature cytokines including IFN-$\gamma$ and TNF-$\alpha$. The cytokine signaling in Th2 cells promotes expression and/or DNA binding of transcription factor GATA3, leading to the synthesis of Th2 signature cytokines such as IL-4, IL-5, and IL-13. The cytokine signaling in Th17 cells promotes expression and/or DNA binding of transcription factor ROR$\gamma$t, leading to the synthesis of Th17 signature cytokines such as IL-17A, IL17F, and IL-21.[37–39] $CD4^+$ naive T cells can also differentiate to iTreg cells, which requires TGF-$\beta$-induced expression of SMAD proteins. SMAD proteins in turn promote the expression of transcription factor Foxp3 that is shared by naturally occurring regulatory T (nTreg) cells derived from DP thymocytes.[40]

We found that RhoA deficiency led to reduced Th2 and Th17 cell differentiation, as evidenced by decreased expression of Th2 and Th17 cell signature cytokines and transcription factors. RhoA deficiency had no effect on Th1, iTreg, and nTreg cell differentiation.[35,41] These findings suggest that RhoA is critical for the differentiation

of Th2 and Th17, but not Th1, iTreg, and nTreg cells. Glycolysis is important for effector T cell differentiation.[42,43] We have found that RhoA promotes Th2 cell differentiation via induction of glycolysis.[35] We have further shown that glycolysis upregulates IL-4R and GATA3 to impact on RhoA-mediated Th2 cell differentiation.[35] Moreover, it appears that RhoA regulates Th2 cell differentiation through its immediate downstream effector ROCK.[35,44] On the other hand, deletion of Cdc42 starting from DN thymocytes enhanced Th1 and Th17 cell differentiation,[31,45] whereas post-thymic deletion of Cdc42 inhibited Th2 cell differentiation,[46] suggesting a developmental stage-specific role of Cdc42 in regulating Th cell differentiation. Deletion of Cdc42 starting from DN thymocytes diminished iTreg cell differentiation and destabilized iTreg cells but seemed not to affect nTreg cell development.[31,45] Treg cell-specific deletion of Cdc42 also did not affect nTreg cell development but decreased peripheral nTreg cell numbers and destabilized nTreg cells.[45] Thus, Cdc42 restrains the differentiation of Th1 and Th17 cells but promotes the differentiation of Th2 and iTreg cells and the homeostasis and stability of nTreg cells. Mechanistically, Cdc42 restrains Th17 cell differentiation through repression of glycolysis, whereas it promotes iTreg and nTreg cell stability through induction of glycolysis.

## RhoA and Cdc42 in effector T cell- and Treg cell-mediated inflammatory diseases

Th2 cells help eliminate parasitic organisms, whereas Th17 cells help eradicate extracellular bacterial and fungal infections. On the other hand, both Th2 and Th17 cells promote allergic airway inflammation that drives the development of asthma, a respiratory disease that affects more than 300 million people worldwide. In their regulation of allergic airway inflammation, Th2 cells promote eosinophilic inflammation, whereas Th17 cells promote neutrophilic inflammation. Glucocorticoids are the most effective and widely used anti-inflammation drugs for the treatment of asthma. Unfortunately, glucocorticoids show excessive toxicity over long-term use. Furthermore, around 5–10% of asthma patients develop severe asthma and are refractory to current therapies.[47–49] Therefore, asthma remains an unmet medical need and understanding of the pathobiology of asthma is warranted.

We found that RhoA deletion in T cells attenuated Th2 cell-mediated allergic airway inflammation in an ovalbumin (OVA)-induced mouse model of asthma. Upon OVA induction, RhoA$^{-/-}$ mice showed a drastic reduction in inflammatory cells, particularly eosinophils, in bronchoalveolar lavage fluids (BALF) and the lungs. Th2 cell cytokines (e.g. IL-4, IL-5, and IL-13) in BALF and other mediators of inflammation including Eotaxin, MUC-5AC and Gob-5 in lung tissue and IgE in serum, were significantly lower in RhoA-deficient mice compared with RhoA-proficient mice.[35] We further found that RhoA deletion affected mixed Th2/Th17 cell-mediated allergic airway inflammation that was induced by house dust mite (HDM), a more physiologically relevant allergen than OVA. We found that while HDM-treated RhoA-proficient mice showed robust eosinophils and neutrophils in BALF, HDM-treated RhoA-deficient mice had markedly reduced eosinophils and neutrophils in BALF. In line with this, lung Th2 and Th17 cells were decreased in HDM-treated RhoA-deficient mice.[41] These findings suggest that RhoA-regulated Th2 and Th17 cell differentiation are essential for asthma development. Similar to RhoA deletion, post-thymic deletion of Cdc42 alleviated OVA-induced allergic airway inflammation,[46] suggesting that Cdc42-mediated Th2 cell differentiation is critical for asthma pathogenesis.

Th17 cells are not only important for asthma development but also essential for ulcerative colitis, a wasting disease representing a chronic disabling disorder.[50,51] Colitis is currently treated by standard medication including corticosteroids, immunosuppressive drugs, and anti-TNF$\alpha$ therapies.[52,53] However, treatment failures often occur,[53] necessitating a better understanding of colitis pathobiology.

Our unpublished data found that RhoA deficiency in T cells impeded colitis development in a mouse model of colitis induced by dextran sulfate sodium (DSS). While RhoA-proficient mice showed massive infiltration of inflammatory cells into the colon upon DSS treatment, RhoA-deficient mice had no apparent inflammatory cell infiltration. Specifically, Th17 cells were reduced in the colon of DSS-treated RhoA-deficient mice, suggesting that RhoA-regulated Th17 cell differentiation is required for colitis development. In contrast to RhoA deletion, Cdc42 deficiency in T cells led to exacerbated colitis in both a mouse model of colitis induced by DSS and that induced by naive T cell transfer into Rag1$^{-/-}$ mice.[45] In both of the mouse models, Cdc42 deficiency caused an increase in Th17 cells in the colon.[45] These findings suggest that Cdc42 in T cells restrains Th17 cell-mediated colitis.

Treg cells play a central role in maintaining immune tolerance by inhibition of effector T cell function. Defects in Treg cell differentiation, homeostasis and stability may lead to excessive effector T cell responses and concomitant autoimmunity.[54,55]

In keeping with the defective differentiation and stability, Cdc42-deficient iTreg cells lost their function in suppressing Th17 cells and Th17 cell-mediated colitis.[45] Consistent with the defective homeostasis and stability, Cdc42-deficient nTreg cells failed to maintain immune tolerance, resulting in increased effector T cells and systemic inflammatory disorders in Treg cell-specific Cdc42 knockout mice.[45] These findings suggest that Cdc42 in Treg cells acts to maintain immune homeostasis and prevent autoimmune diseases.

## Are RhoA and Cdc42 targetable for T cell-mediated inflammatory diseases?

The inhibition of allergy airway inflammation by genetic targeting of RhoA and Cdc42 suggests that RhoA and

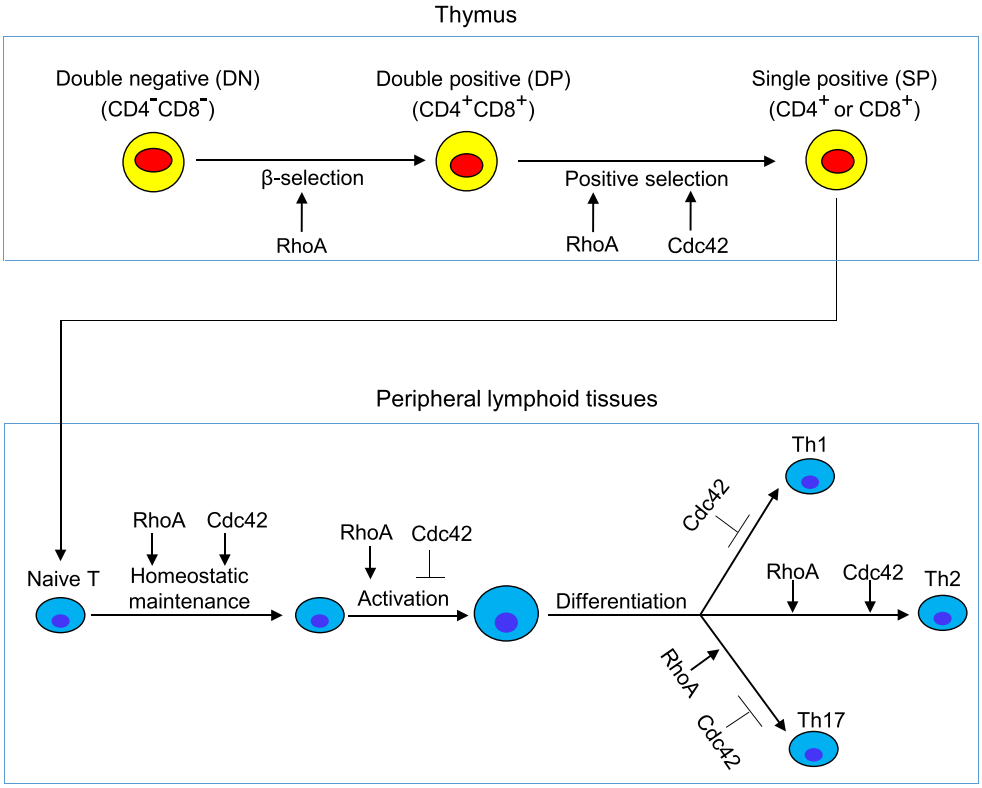


**Figure 1.** Role of RhoA and Cdc42 in thymocyte development and peripheral T cell homeostasis, activation, and differentiation. →: Promotion; ⊣: Inhibition.

Cdc42 are biologic targets for asthma. In support, a RhoA chemical inhibitor Y16 ameliorated HDM-induced allergic airway inflammation and a Cdc42 chemical inhibitor CASIN attenuated both OVA- and HDM-induced allergic airway inflammation.[41,46] The inhibitory effects of Y16 and CASIN on allergic airway inflammation were likely through their effects on Th2 and/or Th17 cell differentiation. As such, 30 $\mu$M of Y16 suppressed Th2 and Th17 cell differentiation *in vitro* and 30 mg/kg of Y16 suppressed Th2 and Th17 cells in a mouse model of allergy airway inflammation,[41] while 10 $\mu$M of CASIN inhibited Th2 cell differentiation *in vitro* and 30 mg/kg of CASIN inhibited Th2 cells in a mouse model of allergic airway inflammation.[46] Of note, similar to RhoA deletion, Y16 did not affect Th1 and iTreg cell differentiation.[41] Similar to post-thymic Cdc42 deletion, CASIN did not affect Th1, Th17, and iTreg cell differentiation.[46] The selectivity of Y16 and CASIN towards Th2 and/or Th17 cells *in vitro* and in allergic airway inflammation suggests that RhoA and Cdc42 are targetable in treating asthma. In further support, RhoA activity is increased in airway biopsies of asthma patients,[56] indicating that targeting of RhoA will likely be a viable approach in asthma therapy. In its suppression of allergic airway inflammation, 30 mg/kg CASIN did not affect thymocyte development and T cell homeostasis. Importantly, treatment of post-thymic Cdc42-deficient mice with 30 mg/kg CASIN did not further inhibit Cdc42 deficiency-attenuated allergic airway inflammation,[46] suggesting that CASIN does not cause off-target effects. Moreover, in inflammation-free C57BL/6 mice, 30 mg/kg of CASIN did not cause autoantibody production, organ damage/systemic inflammation and weight loss (our unpublished data). Nonetheless, Y16 and CASIN could potentially affect other inflammatory cells involved in asthma development, for instance, eosinophils and mast cells, as well as non-inflammatory asthma-mediating lung epithelial cells and smooth muscle cells. In addition, Y16 and CASIN may affect cells that are not involved in asthma pathobiology. Thus, detailed toxicity and pharmacokinetic studies of Y16 and CASIN are warranted.

With respect to colitis, our unpublished study found that RhoA deficiency starting from DN thymocytes attenuated DSS-induced colitis, suggesting that targeting of RhoA may benefit patients with colitis. However, deletion of RhoA starting from DP thymocytes resulted in spontaneous colitis,[33] cautioning RhoA targeting for colitis treatment. Given that Cdc42 deficiency aggravated colitis,[45] a targeting strategy of suppression of Cdc42 will likely be detrimental for patients with colitis, while a Cdc42 activator may be beneficial.

Finally, targeting of RhoA may benefit patients with multiple sclerosis, because RhoA deficiency ameliorated Th17 cell-mediated neuro-inflammation in experimental autoimmune encephalo-myelitis (EAE),[32] a mouse model of human multiple sclerosis.[57]

## Conclusions

Recent work using conditional gene knockout of RhoA and Cdc42 in T cells has found that both RhoA and

Cdc42 are required for thymocyte development and peripheral T cell homeostasis. RhoA promotes but Cdc42 restrains T cell activation. RhoA does not affect but Cdc42 inhibits Th1 cell differentiation. Both RhoA and Cdc42 are important for Th2 cell differentiation. In addition, RhoA promotes but Cdc42 restrains Th17 cell differentiation (Fig. 1). Consistent with the positive role of RhoA in Th2 and Th17 cell differentiation and of Cdc42 in Th2 cell differentiation, RhoA and Cdc42 contribute to the development of allergic airway inflammation. The selectivity of RhoA inhibitor Y16 towards Th2 and Th17 cells and of Cdc42 inhibitor CASIN towards Th2 cells *in vitro* and *in vivo* implicates that targeting of RhoA and Cdc42 holds new promise in treatment for patients with asthma.

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## Conflict of interest

None declared.

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