SMALL GTPASES
2016, VOL. 7, NO. 3, 173–177
http://dx.doi.org/10.1080/21541248.2016.1181698

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COMMENTARY

Isoform-specific targeting of ROCK proteins in immune cells

Alexandra Zanin-Zhorov[a], Ryan Flynn[b], Samuel D. Waksal[b], and Bruce R. Blazar[b]

[a]Kadmon Research Institute, New York, NY, USA; [b]Division of Blood and Marrow Transplantation, Masonic Cancer Center and Department of Pediatrics, University of Minnesota, Minneapolis, MN, USA

ABSTRACT

Rho-associated kinase 1 (ROCK1) and ROCK2 are activated by Rho GTPase and control cytoskeleton rearrangement through modulating the phosphorylation of their down-stream effector molecules. Although these 2 isoforms share more than 90% homology within their kinase domain the question of whether ROCK proteins function identically in different cell types is not clear. By using both pharmacological inhibition and genetic knockdown approaches recent studies suggest that the ROCK2 isoform plays an exclusive role in controlling of T-cell plasticity and macrophage polarization. Specifically, selective ROCK2 inhibition shifts the balance between pro-inflammatory and regulatory T-cell subsets via concurrent regulation of STAT3 and STAT5 phosphorylation, respectively. Furthermore, the administration of an orally available selective ROCK2 inhibitor effectively ameliorates clinical manifestations in experimental models of autoimmunity and chronic graft-vs.-host disease (cGVHD). Because ROCK2 inhibition results in the suppression of M2-type macrophages while favoring polarization of M1-type macrophages, ROCK2 inhibition can correct the macrophage imbalance seen during age-related macular degeneration (AMD). In summary, the exclusive role of ROCK2 in immune system modulation argues for the development and testing of isoform-specific ROCK2 inhibitors for the treatment of inflammatory disorders.

ARTICLE HISTORY
Received 15 April 2016
Revised 18 April 2016
Accepted 19 April 2016

KEYWORDS
age-related macular degeneration; autoimmunity; chronic graft-versus-host disease; immunological balance; inflammation; macrophages; ROCK1; ROCK2; T cells

Rho-associated coiled-coil kinases (ROCKs) play central roles in the actin cytoskeleton organization and regulate a wide range of fundamental cellular functions, such as contractility, adhesion, migration and phagocytosis.[1-4] The two isoforms ROCK1 and ROCK2 are activated by Rho family GTPases and promote actin-myosin mediated contractile force generation via serine-threonine phosphorylation of numerous down-stream targets including myosin light chain (MLC),[5] myosin binding subunit of myosin phosphatase (MYPT),[6] ezrin/radixin/moesin (ERM) proteins[7] and LIM kinase (LIMK).[8] Although ROCK1 and ROCK2 exhibit 65% overall identity and 92% within the kinase domain[9] the question of whether these 2 isoforms have redundant functions remains controversial and is dependent on the cellular system where they are expressed. Using RNA interference, ROCK1 was reported to be critical for stress fiber formation in fibroblasts, whereas ROCK2 controls cortical contractility and phagocytosis.[10] ROCK1 and ROCK2 play distinct roles in the regulation of keratinocyte differentiation and cell detachment.[11] However, extensive study recently published by Kumper et al. demonstrated that ROCK1 and ROCK2 act redundantly in cell cycle progression and tumorigenesis.[12] Therefore, the activity of each ROCK isoforms needs to be evaluated in a cell type- and stimulus-specific manner. Herein, we discuss the role of ROCK1 and ROCK2 in regulation of immune cell function and the potential therapeutic implication of isoform-specific ROCK inhibitors.

Adaptive immune system cells: T-cells and B-cells

ROCK signaling is critical in the coordination and balancing of T-cell-mediated immune responses, including cellular movement, T-cell receptor (TCR) signaling and the acquisition of the appropriate T-cell effector program.[13-16] While increased ROCK activity has been associated with autoimmunity through its capacity to regulate cytoskeletal proteins,[14,16,17] only the ROCK2 isoform was shown to be physiologically activated in $CD4^+$ T-cells under T-helper cells producing IL-17 (Th17) skewing, specifically implicated in regulating of pro-inflammatory cytokines, such as IL-21 and IL-17, and development of autoimmunity in mice.[18] In humans, oral administration of the selective ROCK2 inhibitor KD025 to healthy subjects attenuates the ability of T-cells to secrete both IL-21 and IL-17 in response to stimulation *ex vivo*.[19] KD025 is ATP competitive small molecule inhibitor, which is 100-fold more selective for the ROCK2 over ROCK1 isoform and effectively

CONTACT Alexandra Zanin-Zhorov Alexandra.Zanin-Zhorov@kadmon.com Kadmon Research Institute, 450 East 29th Street, New York, NY 10016, USA.

down-regulates MLC phosphorylation in human T-cells.[20,21] Moreover, ROCK2-dependent regulation of Th17 pathway was mediated through down-regulation of STAT3 phosphorylation, an inducer of pro-inflammatory cytokine responses, as demonstrated by either pharmacological or siRNA-mediated inhibition of ROCK2 expression in human T-cells. Importantly, a recent study by Flynn et al. demonstrated that targeted inhibition of ROCK2 reversed the clinical and immunologic symptoms of an autoimmune-like syndrome, chronic graft-versus-host disease (cGVHD), a complication of allogeneic haematopoietic cell transplantation, in 2 distinct murine models characterized by an immune-mediated fibrosis.[22] These studies further validated a common mechanism of KD025-mediated downregulation of STAT3 phosphorylation in vivo.[22] In addition to the Th17 pathway, STAT3 signaling is critical for development and function of T follicular helper (Tfh) and germinal B-cells, which in the context of cGVHD and secondary lymphoid organs such as the spleen. These two cell subsets cooperate to induce secretion of auto-antibodies that are deposited in tissues and can lead to fibrosis.[23-25] Indeed, the in vivo inhibition of STAT3 phosphorylation by a selective ROCK2 inhibitor in cGVHD mice leads to robust decrease in the percentage of both Tfh and germinal center B-cells, accompanied by reduced splenic cell expression of interferon regulatory factor-4 (IRF4) and RAR-related orphan receptor (RORγt) transcription factors that regulate differentiation of naïve T-cells into T effector cells.[22] Interestingly, although both ROCK1 and ROCK2 are expressed in T-cells, only ROCK2, but not ROCK1 siRNA down-regulates protein levels of pSTAT3, IRF4 and RORγt, supporting the exclusive role of ROCK2 isoform in regulation of pro-inflammatory T-cell responses.[19] Both ROCK1 and ROCK2 siRNAs efficiently inhibited the phosphorylation of the known ROCK target MLC.[19] Further experiments using pharmacological or siRNA-mediated inhibition of ROCK1 and ROCK2 in B-cells are required to define the role of each isoform in regulation of pro-inflammatory B-cell function.

In non-haematopoietic cells, ROCK proteins have been implicated in TGF-β signaling pathway[26] that plays instrumental role in regulation of both pro-inflammatory Th17 cells and an anti-inflammatory T-cell subset that possesses immune suppressive capabilities, regulatory Foxp3$^+$ T cells (Tregs).[27-29] TGF-β induces the activation of ROCK2 in human CD4$^+$ T-cells in a SMAD2/3-independent manner as a part of a non-canonical TGF-β signaling pathway.[19] ROCK2 inhibition leads to the up-regulation of STAT5 phosphorylation that provides critical survival factors for Treg development, expansion and function, followed by a twofold increase in the percentage of Foxp3$^+$ T-cells.[19] Therefore, targeted inhibition of ROCK2 shifts the balance between Th17 cells and Tregs toward a more regulatory/immunosuppressive environment through concurrent regulation of STAT3 and STAT5 phosphorylation.[30] The increase in regulatory T cell subset and STAT5 phosphorylation also was detected in KD025-treated mice in experimental models of autoimmunity and cGVHD.[19,22] Thus, the ROCK2 isoform controls the balance between pro-inflammatory and anti-inflammatory signaling pathways in T cells.

Innate immune cells: Monocytes and macrophages

Monocyte migration and infiltration into inflamed tissue requires a coordinated remodeling of the actin cytoskeleton.[31] By using a pan-ROCK inhibitor Y27632, it was demonstrated that both ROCK proteins are essential in regulation of monocytes trans-endothelial migration[32] as well as being implicated in controlling of monocyte phagocytosis-dependent IL-1β secretion.[33] Selective ROCK1 ablation leads to increased recruitment and migration of macrophages in both in vitro and in vivo settings via mechanism that involves regulation of the phosphorylation and stability of phosphatase and tensin homolog (PTEN).[34] Interestingly, enhanced migration resulting from ROCK1 deficiency was observed despite normal expression of ROCK2 and a significant reduction in overall ROCK activity. Also, ROCK activity is required for induction of PTEN phosphatase activity and regulation of PTEN intracellular localization during leukocyte chemotaxis.[35] Moreover, a recent study has demonstrated that ROCK1 and ROCK2 play different roles in regulation of macrophages polarization into classical pro-inflammatory IL-12 producing macrophage type 1 (M1) and alternative anti-inflammatory, IL-10 and TGFβ producing, pro-fibrogenic macrophage type 2 (M2) subtypes in age-related macular degeneration (AMD).[36] Selective ROCK2 inhibition decreased M2-like macrophages that are accumulated in and associated with pathogenesis of AMD. In turn, ROCK2, but not dual ROCK1/2 inhibition increased M1 markers and furthered M1 polarization that is beneficial to preserve retinal health. Although ROCK2 targeting (pharmacological and genetic knockdown) does not interfere with overall macrophage recruitment, selective ROCK2 inhibition reduced AMD pathology and restored the immunological balance between M1 and M2 macrophages that prevails in the normal young eye.[36]

Therapeutic implications of isoform-specific ROCK targeting

Since ROCKs play a central role in the organization of the actin cytoskeleton, it might be anticipated that the

Table 1. Outcomes of isoform-specific ROCK targeting in immune cells.

Cell type	ROCK isoform	Cell function/effect of ROCK inhibition	Preclinical model/outcomes	Ref.
T cells	ROCK1/2	Adhesion and Migration/Inhibition	SLE patients-derived T cells	14,16
T cells	ROCK1/2	Proliferation/Inhibition	Increased survival after allogeneic heart transplantation in mice	15
Th17 cells	ROCK2	IL-17 and IL-21 secretion, pSTAT3/ Inhibition	Collagen-Induced arthritis and cGVHD model/Inhibition	18,19,22
Tfh cells	ROCK2	Percentage and function of $CXCR5^{+}PD1^{+}$ Tfh cells/Inhibition	cGVHD and sclerodermatous GVHD model/Inhibition	22
Treg cells	ROCK2	Percentage of Foxp3, pSTAT5, Treg function/Increase	Collagen-Induced arthritis and cGVHD model/Inhibition	19,22
Macrophages	ROCK2	M2 polarization/Inhibition M1 polarization/Increase	Restoration of normal macrophage balance in AMD	36
Macrophages	ROCK1/2	Migration in MCP-1 implanted corneas/ Inhibition	Restoration of normal macrophage balance in AMD	36
Monocytes	ROCK1/2	Asbestos-induced IL-1b secretion/ Inhibition	In vitro stimulation of THP-1/Inhibition	33
Macrophages	ROCK1	Migration/Increase	Wound-healing in vitro assay and cell migration in vivo/Increase	34

complete inhibition of both isoforms could cause adverse events in patients.[37] Therefore, selective ROCK2 inhibition could have a greater specific therapeutic index over the dual inhibition of ROCK1 and ROCK2. Indeed, a placebo-controlled, randomized, phase1 clinical study showed that the the oral administration of the selective ROCK2 inhibitor, KD025, was well tolerated, without significant adverse events in healthy human subjects.[19] The maximum concentration of the drug in peripheral blood was linear and dose proportional at doses of 40-500 mg. Moreover, an analysis of peripheral blood mononuclear cells revealed that KD025 effectively down-regulated the secretion of pro-inflammatory cytokines, IL-21 and IL-17 during stimulation ex vivo, which is consistent with the exclusive role of ROCK2 isoform in regulation of Th17 pathway. In addition, the antagonism of ROCK1 and ROCK2 isoforms in the regulation of macrophage polarization suggests that more efficacy and fewer side effects are possible with selective ROCK2 targeting compared to dual ROCK1/2 inhibition in immune-cell mediated pathologies, such as autoimmunity, cGVHD and AMD (Table 1).

In conclusion, we are at an early stage of evaluating the unique role of ROCK1 and ROCK2 isoforms in the regulation of immune cell function both in steady state as well as pathological conditions. Whereas dual ROCK1/2 inhibition has a profound effect on cytoskeletal proteins and leukocyte migration, selective ROCK2 inhibition targets cell plasticity and efficiently restores the balance between pro-inflammatory and regulatory phenotype of immune cells (Fig. 1). Thus, targeted ROCK2 inhibition

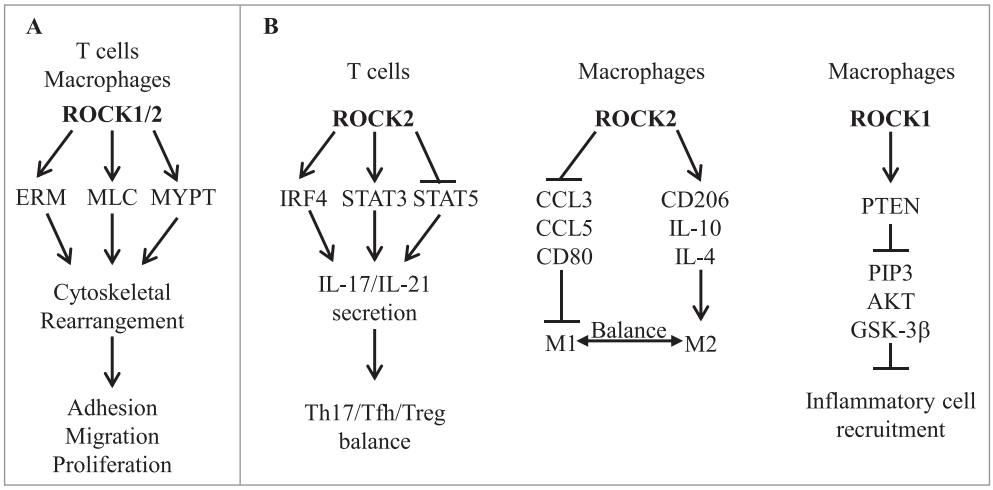

Figure 1. Typical (A) and isoform-selective (B) signaling pathways downstream of ROCK proteins. ERM, ezrin/radixin/moesin; MLC, myosin light chain; MYPT, Myosin binding subunit of myosin phosphatase; IRF4, IFN regulatory factor 4; STAT3/5, signal transducer and activator of transcription 3/5; Th17, T helper 17; Tfh, T follicular helper; Treg, regulatory T cell; M1 and M2, M1-type and M2-type of macrophages; PTEN, phosphatase and tensin homolog; PIP3, phosphatidylinositol 3,4,5-triphosphate; AKT, protein kinase B; GSK-3β, Glycogen synthase kinase 3 β.

represents a novel therapeutic approach for treatment of inflammatory disorders.

Disclosure of potential conflicts of interest

Alexandra Zanin-Zhorov is an employee of Kadmon; Samuel D. Waksal is founder of Kadmon.

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