

EXTENDED REPORT

Targeting the RhoA-ROCK pathway to reverse T-cell dysfunction in SLE

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ABSTRACT

Objectives Deregulated production of interleukin (IL)-17 and IL-21 contributes to the pathogenesis of autoimmune disorders such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA).

Production of IL-17 and IL-21 can be regulated by ROCK2, one of the two Rho kinases. Increased ROCK activation was previously observed in an SLE cohort. Here, we evaluated ROCK activity in a new SLE cohort, and an RA cohort, and assessed the ability of distinct inhibitors of the ROCK pathway to suppress production of IL-17 and IL-21 by SLE T cells or human Th17 cells.

Methods ROCK activity in peripheral blood mononuclear cells (PBMCs) from 29 patients with SLE, 31 patients with RA and 28 healthy controls was determined by ELISA. SLE T cells or in vitro-differentiated Th17 cells were treated with Y27632 (a pan-ROCK inhibitor), KD025 (a selective ROCK2 inhibitor) or simvastatin (which inhibits RhoA, a major ROCK activator). ROCK activity and IL-17 and IL-21 production were assessed. The transcriptional profile altered by ROCK inhibitors was evaluated by NanoString technology.

Results ROCK activity levels were significantly higher in patients with SLE and RA than healthy controls. Th17 cells exhibited high ROCK activity that was inhibited by Y27632, KD025 or simvastatin; each also decreased IL-17 and IL-21 production by purified SLE T cells or Th17 cells. Immune profiling revealed both overlapping and distinct effects of the different ROCK inhibitors.

Conclusions ROCK activity is elevated in PBMCs from patients with SLE and RA. Production of IL-17 and IL-21 by SLE T cells or Th17 cells can furthermore be inhibited by targeting the RhoA-ROCK pathway via both non-selective and selective approaches.

INTRODUCTION

Aberrant expansion and dysregulation of several T_H effector subsets including Th17 and T_H17 cells can be associated with autoimmune disorders such as systemic lupus erythematosus (SLE) and rheumatoid Arthritis (RA).^{1–4} The pathogenicity of these cells has been linked to their ability to produce cytokines such as interleukin (IL)-17 and IL-21. Several transcription factors have been implicated in the regulation of Th17 differentiation.⁵ Notably, Th17 differentiation and the production of IL-17 and IL-21 are critically dependent on the presence of IRF4, a member of the interferon regulatory factor (IRF) family of transcription factors.^{6–8} The ability of IRF4 to drive high levels of IL-17 and

IL-21 production can be regulated via its phosphorylation by ROCK2.⁹ ROCK2 and the other member of the Rho kinase family, ROCK1, are two serine/threonine kinases whose activity is primarily controlled by binding of activated RhoA.^{10–13} The RhoA-ROCK pathway regulates multiple biological functions, including cytoskeletal reorganisation, proliferation and differentiation.^{10–13}

While dysregulated activation of the RhoA-ROCK pathway in non-haematopoietic cells is known to contribute to cardiovascular, renal and neurological disorders,^{14–16} recent work has also implicated aberrant ROCK activation in the pathogenesis of autoimmune disorders.¹⁷ In line with the ability of ROCK2 to control IRF4 function and the production of IL-17 and IL-21, ROCK2 is selectively activated in murine and human Th17 cells and aberrant ROCK2 activation has been observed in murine lupus T cells.^{9–18} Furthermore, the administration of a pan-ROCK inhibitor, Fasudil, diminishes IL-17 and IL-21 production in vivo and ameliorates disease in spontaneous murine models of lupus.^{9–19} Consistent with these observations, SLE T cells, including T cells infiltrating the kidneys, exhibit increased phosphorylation of ezrin, radixin, moesin (ERM) proteins, a ROCK target and the ROCK-mediated effects can be promoted by protein phosphatase 2A (PP2A), a phosphatase expressed at higher levels in SLE T cells than T cells from healthy controls.^{20–21} An initial pilot study, furthermore, directly demonstrated enhanced ROCK activity in peripheral blood mononuclear cells (PBMCs) from ~60% of patients with SLE suggesting that inhibition of this pathway represents a potential therapeutic target for SLE and potentially other autoimmune diseases such as RA.²²

The attractiveness of the RhoA-ROCK pathway as a therapeutic target for SLE is further strengthened by the availability of a wide array of ROCK inhibitors.^{23–27} Most ROCK inhibitors, such as the well-known Fasudil and Y27632, target the ATP-binding pocket of the ROCKs and, due to the high degree of homology in the kinase domain of ROCK1 and ROCK2, are thus non-isoform selective. Selective oral ROCK2 inhibitors, such as KD025 (formerly known as Slx-2119) that demonstrates 100-fold more selectivity towards ROCK2 than ROCK1, have, however, also been developed.^{18–28} Inhibition of ROCK activation is also a key mechanism underlying the pleiotropic effects of statins since by inhibiting HMG-CoA reductase, statins interfere with RhoA activation and,



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consequently, decrease ROCK activity.²⁹ Here, we systematically compared the ability of different classes of ROCK inhibitors to decrease IL-17 and IL-21 production by either SLE T cells or in vitro-differentiated human Th17 cells. In addition, we evaluated the broader transcriptional effects of these different inhibitors on human Th17 cells by NanoString technology.

METHODS

Human subjects

SLE subjects

Twenty-nine patients with SLE followed at the Hospital for Special Surgery (HSS) were consented and enrolled. Inclusion criteria were as follows: age 18–65 years and classification of SLE according to the revised 1997 American College of Rheumatology (ACR) criteria.³⁰ Exclusion criteria were as follows: statin use, active infection by history, pregnancy, pulse glucocorticoids within 1 month, cyclophosphamide within 3 months, rituximab within 1 year and any anti-tumor necrosis factor (TNF) therapy within 3 months. Clinical data including laboratory parameters, medications, complications and serology were collected. Disease activity was assessed at time of sampling blood by the Systemic Lupus Erythematosus Disease Activity Index Safety of Estrogen in Lupus Erythematosus National Assessment (SELENA) modification (SELENA-SLEDAI).³¹ Five of 29 patients with SLE were taking ACE inhibitors or angiotensin II receptor blockers (ARBs).

RA subjects

Thirty-one patients with RA from HSS were consented and enrolled. Inclusion criteria: age >18 years and meeting ACR/European League Against Rheumatism 2010 RA classification criteria.³² Exclusion criteria were as follows: active infection, pregnancy, recent surgery, history of Raynaud or another autoimmune diagnosis. Clinical data including laboratory parameters, medications, complications and serology were collected. Disease activity was assessed at time of sampling using the Clinical Disease Activity Index.³³ Radiographs of hands and wrists were also reviewed, if available, to determine presence or absence of erosions. Patients with RA were treated at the discretion of their doctor, and specific medications included one or more of the following: hydroxychloroquine, leflunomide, methotrexate, etanercept, adalimumab, systemic corticosteroids and non-steroidal anti-inflammatory drugs. Four of 31 patients with RA were taking ACE inhibitors or ARBs.

Healthy controls

Twenty-four sex-age-ethnically similar healthy controls were consented and enrolled at HSS. Four healthy controls samples were provided by the Genotype and Phenotype Registry, a service of the Tissue Donation Program at The Feinstein Institute for Medical Research, Manhasset, New York, USA (supported by NIH grant SRC2AR05092). Healthy controls were age >18 years. Exclusion criteria were as follows: active infection, pregnancy, recent surgery, history of Raynaud or another known diagnosis of an autoimmune disease. Of the healthy controls, 9 matched by age (± 5 years), sex and ethnicity to the patients with SLE, 13 matched to the patients with RA and 6 matched to both SLE and RA.

The HSS Institutional Review Board approved the study, and all blood donors provided written informed consent.

Samples

Peripheral blood mononuclear cells

Heparinised venous blood samples (10–20 mL) were obtained by hospital phlebotomists and processed immediately. PBMCs were isolated from each subject, whole cell extracts obtained and then frozen. At a later date, extracts were thawed and ROCK assays performed on groups of samples.

T-cell cultures

SLE CD4⁺ T cells were negatively selected by MACS (Miltenyi). Cells were plated at 2×10^5 cells/well in X-VIVO15 media (Lonza) and stimulated with soluble α CD28 (1 μ g/mL) and plate-bound α CD3 (5 μ g/mL; OKT3 clone) for 4 days. In separate experiments, cryopreserved human cord blood CD4⁺ T lymphocytes from different donors were obtained from Allcells, LLC. CD4⁺ T-cell purity was >95%. Cells were thawed according to the manufacturer's instructions and cultured as described above. For Th17 differentiation, medium was supplemented with transforming growth factor (TGF)- β 1 (5 ng/mL), IL-1 β (10 ng/mL), IL-6 (20 ng/mL) and IL-23 (50 ng/mL). Y27632 60–90 μ M (EMD), KD025 5 μ M (provided by Kadmon) and simvastatin 0.1–0.2 μ M (Calbiochem) were added at day 1. Supernatants were analysed for IL-17 (Biolegend), IL-21 (eBioscience), Chemokine (C-C motif) ligand (CCL20) (R&D Systems) and interferon (IFN)- γ (Biolegend) levels by ELISA.

ROCK activity assays and western blotting

ROCK activity in extracts was measured using the 96-well ROCK Activity Assay Kit (Cell Biolabs) as described.²² Active ROCK2 (1–4 ng) served as a positive control. IRF4 phosphorylation was detected with a phospho-IRF4 Ab⁹ and total levels of IRF4 with a goat anti-IRF4 antibody (M-17; Santa Cruz Biotechnology). Blots were also probed for either phospho-Stat3 (Y705; #9131; Cell Signaling) or total Stat3 (Cat: 610189; BD Transduction Labs).

RESULTS

Increased ROCK kinase activity in SLE and RA PBMCs compared with healthy control (HC) PBMCs

In an initial cross-sectional study, we had observed that ~60% of patients with SLE exhibited increased ROCK kinase activity

Table 1 Summary of demographic data and patient characteristics

Parameter	Patients with SLE n=29	Patients with RA n=31	Healthy controls n=28
Mean age, years \pm SD	38 \pm 11	59 \pm 14	46 \pm 15
Females, n (%)	27 (93)	27 (87)	25 (89)
Males, n (%)	2 (7)	4 (13)	3 (11)
Ethnicity			
African-American, n (%)	9 (31)	4 (13)	4 (14)
Asian, n (%)	2 (7)	6 (19)	3 (11)
Caucasian, n (%)	5 (21)	17 (55)	13 (46)
Hispanic, n (%)	12 (41)	4 (13)	8 (29)
SLEDAI \pm SD	6 \pm 4		
Nephritis, n (%)	13 (45)		
Mean ESR (mm/hour) \pm SD		28.0 \pm 21.3	
Mean CRP (mg/dL) \pm SD		2.16 \pm 3.92	
CDAI, mean \pm SD		13.8 \pm 7.52	

CDAI, Clinical Disease Activity Index; CRP, c-reactive protein; ESR, Erythrocyte Sedimentation Rate; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

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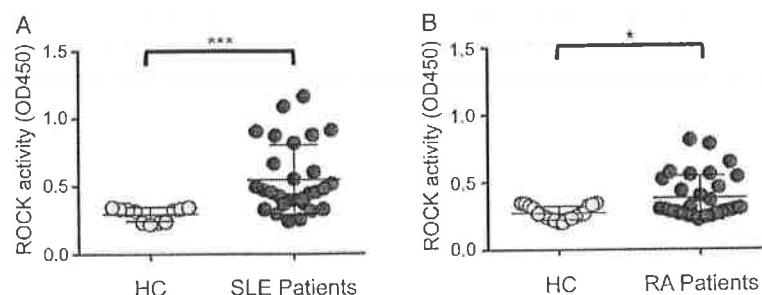


Figure 1 Increased ROCK kinase activity in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) peripheral blood mononuclear cells (PBMCs) compared with HC PBMCs. PBMCs were isolated from heparinised blood samples of 29 patients with SLE (A) and 31 patients with RA (B). PBMCs from healthy controls of similar ethnicity/race, sex and age \pm 5 years (table 1) for each of the disease group were also assayed. Whole cell extracts were prepared, and ROCK activation was assessed by an ELISA-based ROCK kinase activity assay. Active ROCK2 (1–4 ng) was used as a positive control. Data were analysed by the Kruskal-Wallis test ($\chi^2=26.25$, $p<0.0001$) followed by the Dunn's multiple comparison analysis (HC vs RA $p=0.087$, RA vs SLE $**p<0.01$, HC vs SLE $***p<0.001$).

as compared with healthy controls.²² To confirm these findings, we investigated ROCK kinase activity in a second SLE cohort (table 1). ROCK activity was significantly elevated in SLE PBMCs as compared with PBMCs from healthy controls (0.457 (IQR: 0.368–0.743) vs 0.278 (IQR: 0.230–0.332) respectively, $p<0.0001$, Mann Whitney (MW)) (figure 1A). PBMCs from ~70% of patients with SLE from this second cohort displayed ROCK activity levels that were 2 SD above the mean of the ROCK activity levels observed in PBMCs from healthy controls. Thus, patients with SLE from two independent cohorts demonstrate increased ROCK activity.

To assess whether elevated ROCK activation was specific for SLE or could also be observed in another autoimmune disease, we measured ROCK activity levels in PBMCs from 31 patients with RA and 19 healthy controls of similar demographics with respect to age, gender and race/ethnicity (table 1). As compared with PBMCs from healthy controls, PBMCs from patients with RA also expressed significantly higher median ROCK activity levels (0.299 (IQR: 0.264–0.522) vs 0.259 (IQR: 0.229–0.324) respectively, $p<0.015$, MW) (figure 1B) although the extent of ROCK activation in RA PBMCs was not as great as that observed in some of the SLE PBMCs.

Effects of different ROCK inhibitors on the production of IL-17A and IL-21 by SLE T cells

Cytokine production by cord blood-derived Th17 cells can be decreased by Y27632, a non-selective ROCK inhibitor.²² To assess whether production of IL-17 and IL-21 by SLE T cells is also ROCK dependent, we next purified CD4⁺ T cells from PBMCs of patients with SLE and stimulated them \pm Y27632 (figure 2A–C). Given that ROCK activity can also be inhibited by statins via effects on RhoA, we also compared the capacity of Y27632 and simvastatin to decrease the production of IL-17A and IL-21 by SLE T cells (figure 2A–C). Both Y27632 (at 90 μ M) and simvastatin (at 0.2 μ M) diminished the production of IL-17A and IL-21 by SLE T cells, while no significant effects were observed on the production of IFN- γ (figure 2A–C). Since simvastatin and Y27632 interfere with ROCK activation by different mechanisms, we also evaluated the effect of combining Y27632 and simvastatin (figure 2A, C). Addition of simvastatin to the lower dose of Y27632 (30 μ M) did not further increase the inhibition of IL-17A or IL-21 production (figure 2A, C). Thus, both simvastatin and the non-selective ROCK inhibitor decrease the production of IL-17A and IL-21 by SLE T cells.

In view of the recent availability of a selective ROCK2 inhibitor, KD025, we next compared the effects of KD025 with those of the pan-ROCK inhibitor, Y27632, and simvastatin on the production of IL-17A and IL-21. The ROCK2 selective inhibitor was also effective in diminishing the production of IL-17A ($t=-2.57$, $p=0.014$) and IL-21 ($t=-4.08$, $p=0.0004$). No effects on the production of IFN- γ were again observed ($t=-0.27$, $p=0.7827$). Thus, the production of IL-17A and IL-21 by SLE T cells is broadly amenable to inhibition by different ROCK inhibitors.

Effects of different ROCK inhibitors on cytokine production by differentiated Th17 cells

To evaluate whether different classes of ROCK inhibitors could also interfere with the production of IL-17A and IL-21 by non-autoimmune T cells differentiated under Th17 conditions, we first compared the capacity of Y27632 and simvastatin to decrease the production of IL-17A, IL-21 and CCL20 by cord blood CD4⁺ T cells cultured under Th17-skewing conditions (figure 3A, C). Consistent with our prior results,²² Y27632 at 90 μ M was highly effective in inhibiting IL-17A production ($t=-5.59$, $p<0.0001$, mixed linear modeling (MLM), figure 3A–C, supplementary figure s1). 60 μ M of Y27632 could also inhibit IL-17A production albeit to a lesser degree ($t=-2.58$, $p=0.016$, MLM, figure 3A–C). As previously observed,²² IL-21 production was also sensitive to the inhibitory effects of Y27632 and both 60 μ M of Y27632 ($t=-3.32$, $p=0.004$) and 90 μ M of Y27632 ($t=-4.96$, $p<0.0001$) were highly effective in diminishing IL-21 levels (figure 3A–C). The production of CCL20 was also decreased by both doses of Y27632 (60 μ M: $t=-2.6$, $p=0.016$; 90 μ M: $t=-4.49$, $p=0.0002$) although this effect was less marked and the variance was larger compared with the inhibitory effects exerted on IL-21 production (figure 3A–C).

In comparison with Y27632, simvastatin (0.1 μ M) alone did not decrease IL-17A ($t=-1.11$, $p=0.27$) or CCL20 production ($t=1.9$, $p=0.06$), while the effect on IL-21 production was more variable (figure 3A, C). Higher doses of simvastatin exerted toxic effects in this cell culture system and could not be evaluated (data not shown). As observed for SLE T cells, the addition of simvastatin to the lower dose of Y27632 did not further increase the inhibition of CCL20 or IL-21 production but enhanced the ability of the lower dose of Y27632 to decrease IL-17A production (figure 3C). We did not detect any significant inhibitory effects of either Y27632 or simvastatin on the levels of IRF4 mRNA indicating that the effects of the

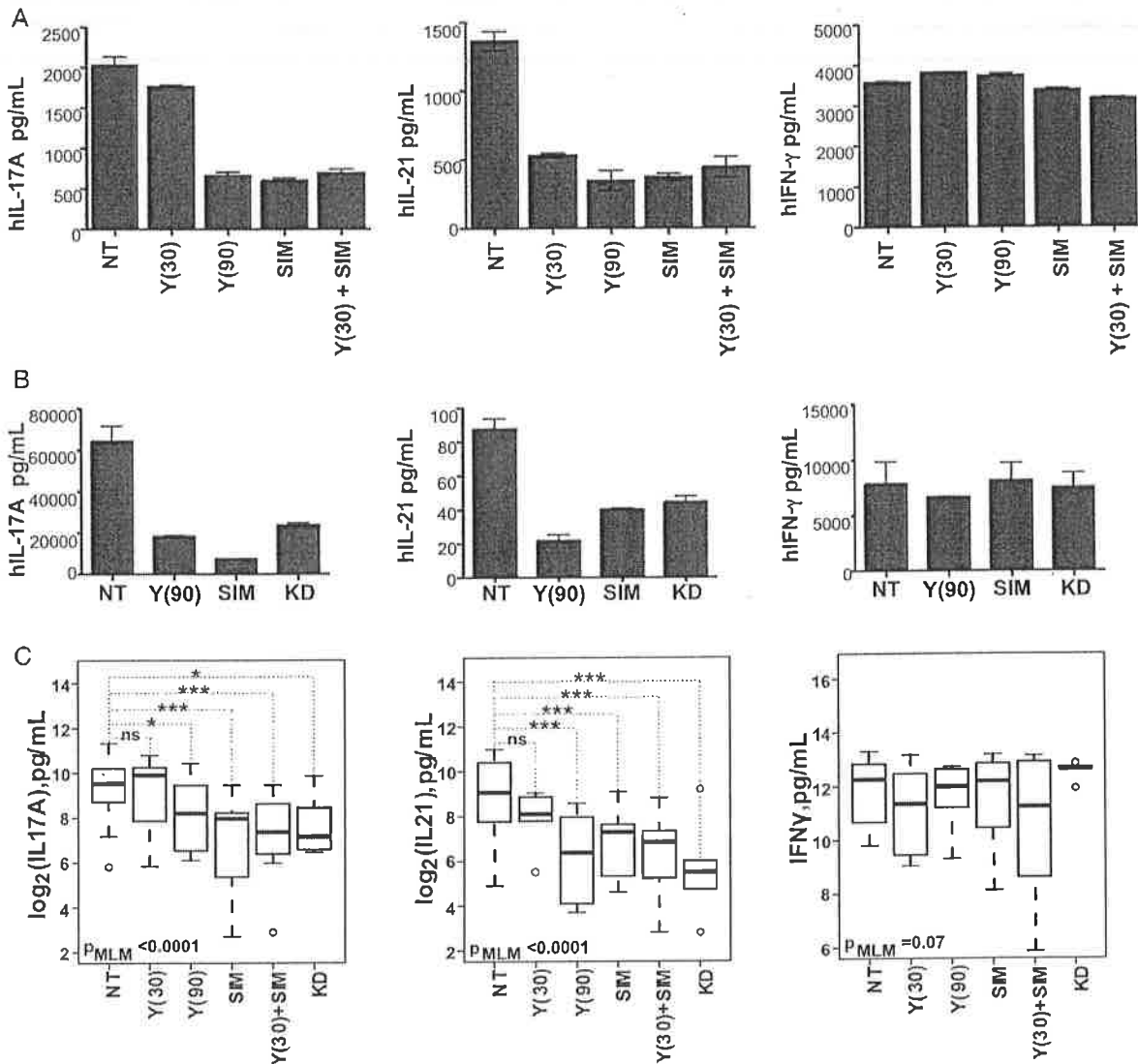


Figure 2 Decreased hIL-17A, hIL-21 and hIFN- γ production by systemic lupus erythematosus (SLE) CD4⁺ T cells treated with ROCK inhibitors. CD4⁺ T cells were isolated from SLE PBMCs and cultured with plate-bound anti-CD3 and anti-CD28 stimulation in X-VIVO15 media. One day later, CD4⁺ T cells were treated with (A) Y27632, simvastatin or Y27632 plus simvastatin or (B) Y27632 or simvastatin or KD025. Supernatants and cells were collected on day 4 and supernatant levels of hIL-17, hIL-21 and hIFN- γ production were assessed. Shown is one representative experiment. (C) The inhibition of hIL-17A, hIL-21 and hCCL20 production by each inhibitor and the combination thereof was fitted to a mixed linear model. p Values from the likelihood ratio test comparing the null model with only the random effect of patients with the complete model that includes all treatments are shown on each panel. The p values for individual treatment were calculated as described in the Materials and Methods section: Y(30), 30 μ M Y27632; Y(90), 90 μ M Y27632; SIM, 0.2 μ M simvastatin; KD, 5.0 μ M KD025.

inhibitors are not due to decreased IRF4 transcription (see online supplementary figure S2A). Thus, the non-selective ROCK inhibitor was more effective than simvastatin alone in modulating the cytokine profile of in vitro-differentiated Th17 cells.

We next compared the effects of KD025 with those of the pan-ROCK inhibitor, Y27632, on cytokine production by Th17 cells. KD025 (5 μ M) was as effective as the higher dose of Y27632 in inhibiting the production of IL-17A ($t=-7.1$, $p<0.0001$), IL-21 ($t=-3.9$, $p=0.0008$) and CCL20 ($t=-6.58$, $p<0.0001$) and more effective than the lower dose of Y27632 at decreasing IL-17A and CCL20 production (figure 3B, C). When assessing IRF4 as a measure of cell viability and T-cell stimulation, we did not observe any inhibitory effect on IRF4 gene expression in Th17 cells treated with either the non-

selective ROCK inhibitor or the ROCK2 selective inhibitor (see online supplementary figure S2B). To confirm that the effects of the different inhibitors on cytokine production were linked to effects on ROCK activation, we performed an ELISA-based ROCK kinase assay (figure 3D). Consistent with the finding that the higher levels of ROCK activity observed in Th17 cells than in Th0 cells are primarily due to ROCK2 activation,⁹ the ROCK2 selective inhibitor was as effective as the non-selective ROCK inhibitor or simvastatin at diminishing ROCK activity (figure 3D). In line with these results, all three inhibitors diminished the phosphorylation of IRF4, a known target of ROCK2 (figure 3E). Neither Y27632 nor KD025 decreased Stat3 phosphorylation (figure 3E). Therefore, a selective ROCK2 inhibitor is as effective as a non-selective ROCK inhibitor at decreasing cytokine production by in vitro-differentiated Th17 cells.

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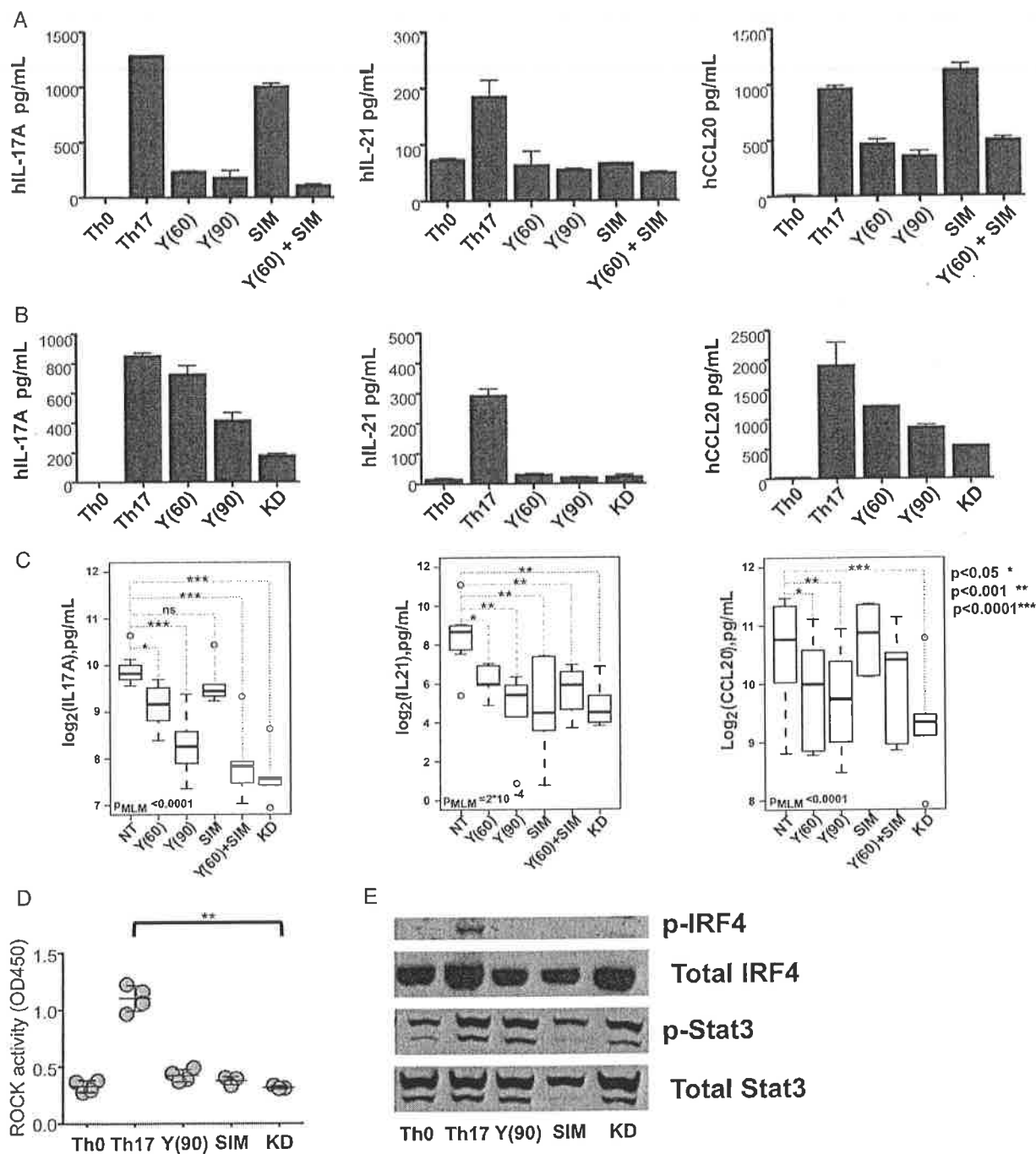


Figure 3 ROCK inhibitors decrease hIL-17A, hIL-21 and hCCL20 production by in vitro-differentiated Th17 cells. Human umbilical cord CD4⁺ T cells (10^6 cells/well) were cultured under Th0 or Th17-polarising conditions for 2 days and then treated with (A) Y27632, simvastatin or Y27632 plus simvastatin or (B) Y27632 or KD025. Supernatants and cells were collected on day 4 and supernatant levels of hIL-17A, hIL-21 and hCCL20 were assessed. Shown is one representative experiment. (C) The inhibition of hIL-17A, hIL-21 and hCCL20 production by each inhibitor and the combination thereof was fitted to a mixed linear model. p Values from the likelihood ratio test comparing the null model with only the random effect of patients with the complete model that includes all treatments are shown on each panel. The p values for individual treatment were calculated as described in the Materials and Methods section. (D) Whole cell extracts were prepared and ROCK activity was measured. Shown are three to four independent experiments. Data were analysed by the Kruskal-Wallis test ($\chi^2=13.48$, $p<0.009$) followed by the Dunn's multiple comparison analysis (** $p<0.01$). (E) Phosphorylation of Stat3 protein was assessed by western blotting, blots were stripped and reprobed for total Stat3, and total IRF4, respectively. Y(60), 60 μ M Y27632; Y(90), 90 μ M Y27632; SIM, 0.1 μ M simvastatin; KD, 5.0 μ M KD025. IRF, interferon regulatory factor.

Immune profiling reveals both overlapping and distinct effects of different ROCK inhibitors

To gain a broader understanding of the regulatory effects of the different classes of ROCK inhibitors in Th17 cells, we employed

the NanoString nCounter technology to profile the expression of immune-relevant genes. Human immunology V2 CodeSet contains probe sets recognising 581 immuno-relevant target genes (http://www.nanostring.com/support/literature?cat=gene_

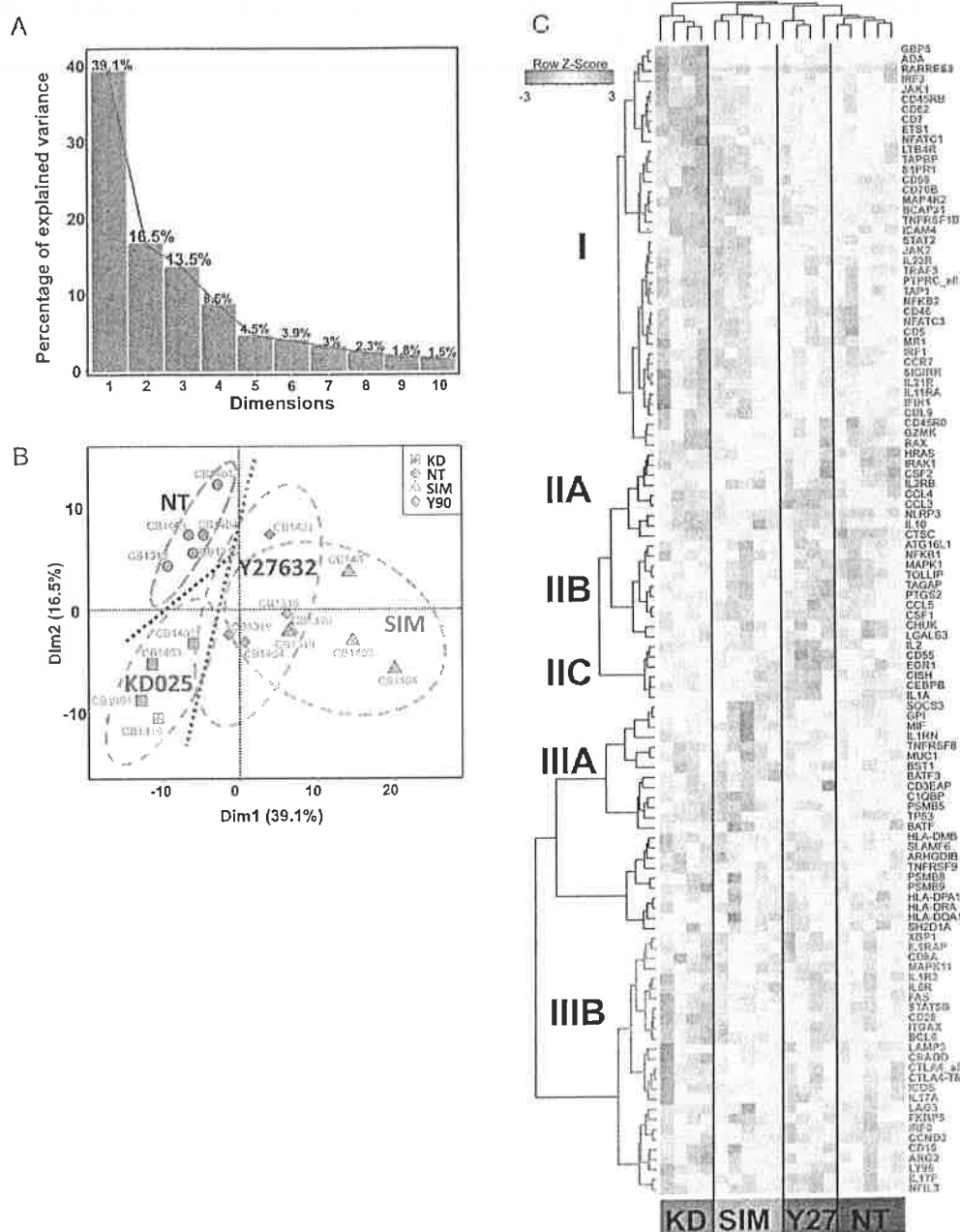


Figure 4 KD025 and Y27632 regulate distinct subset of immune-relevant genes. Following preliminary low-stringency NanoString screening for genes differentially expressed in Th17 cells treated with KD025 and Y27632, we performed principal component analysis of independent cell samples (the sample coding is shown next to the data points) with gene expression as variables. (A) The first four principal components (PCs) explain 77.7% of variance. (B) The first PC separates Y27632/SIM-treated Th17 cells from untreated (NT) and KD025-treated Th17 cells, whereas the second PC separates KD025-treated and NT cells. (C) Unsupervised hierarchical clustering was used to define the group of coregulated genes in Th17 cells following treatments with Y27632, SIM or KD025 in comparison with untreated cells. Genes specifically downregulated in KD025-treated cells (cluster I) are shown in red, cluster IIC that contains a group of genes specifically downregulated by Y27632 is shown in blue and cluster IIIB combines genes downregulated in Th17 cells treated with either Y27632 or KD025.

lists). Two hundred and twenty-five genes passed initial screen for differential expression (see online supplementary methods) and were used for further analysis. Principal component analysis (R, *FactoMineR*)³⁴ was used to investigate the underlying data structure of expression profiling data set containing 225 genes and 18 treatment conditions. The log-transformed expression count values were standardised prior to the analysis. The first four principal components (PC) explained 39.1%, 16.5%, 13.5% and 8.6% of the variance, respectively (figure 4A, see

online supplementary figure S3A–C). Contribution of individual genes to each of the first three PCs was calculated as ratio of the squared factor score of a gene by the eigenvalue associated with that component using *FactorMineR*. Genes with the above average contribution for a given PC (PC1-112, PC2-73, PC2-71 gene, see online supplementary figure S3) were selected for further analysis. The PC1 separates groups of genes with similar regulation in response to either simvastatin or Y27632 (figure 4B and see online supplementary figure S3A), indicating that these two compounds

Basic and translational research

act on similar immune-related regulatory pathways. The PC2 effectively separates genes altered in KD025-treated cells (figure 4B and see online supplementary figure S3B) and PC3 is primarily driven by the differences between Y27632 and other treatments (see online supplementary figure S3C).

Hierarchical clustering of genes differentially regulated by Y27632, KD025 and simvastatin in comparison with untreated cord blood-derived Th17 ($p < 0.05$) revealed that all inhibitors downregulated a small number of genes, albeit to different extents (figure 4C, cluster IIIB). In addition, KD025 specifically inhibited a subset of genes (cluster I), which was either largely unaffected or only weakly downregulated by Y27632 or simvastatin relative to untreated cells. Notably, KD025 downregulated the expression of both IL-23R and IL-21R (cluster I). The gene profile affected by Y27632 and simvastatin was instead largely overlapping (figure 4C and see online supplementary figure S3A) except for a small group of genes (cluster IIC) that were primarily inhibited by Y27632 but not simvastatin or KD025 and that included IL-2. Finally, a small group of genes was upregulated by both KD025 and simvastatin (clusters IIA and IIB) but not Y27632.

Comparative analysis of regulatory pathways enriched in KD025-responsive versus Y27632-responsive genes (both up and down) using G:profiler³⁵ further indicated that both inhibitors controlled a number of genes involved in the regulation of cytokine production (see online supplementary figure S4). In addition, Y27632 regulates a battery of unique genes that are involved in leucocyte differentiation and cell adhesion, viral response and interactions between lymphoid and non-lymphoid cells. Furthermore, treatment with Y27632 is associated with induction of several elements of the interferon signalling pathway. KD025 instead altered the expression of genes involved in signal transduction downstream of multiple transmembrane receptors (figure 4C and see online supplementary figure S4). Specifically, KD025 treatment downregulates several elements of the Jak-STAT pathway as well as several subunits of the immunoproteasome. Interestingly, Y27632 and KD025 potentially exert opposite effects on the same pathways. For example, while KD025 decreases mRNA levels for JAK1, JAK2, IRF3 and STAT5B, Y27632 upregulates the expression of these genes (see online supplementary figure S4).

DISCUSSION

The ROCKs are emerging as important regulators of T-cell effector function via their ability to be activated under Th17 conditions and to regulate the production of IL-17 and IL-21 and initial studies revealed that ~60% of patients with SLE exhibit increased ROCK activity in their PBMCs.²² Here we have investigated ROCK activation in a new cohort of patients with SLE and have confirmed that PBMCs from a majority of patients with SLE exhibit higher levels of ROCK activity than healthy controls. Interestingly, PBMCs from patients with RA were also found to exhibit higher ROCK activity levels than controls suggesting that dysregulated ROCK activation may represent a common pathogenic pathway in autoimmunity.

Given the potential of the ROCKs as therapeutic targets for SLE and other autoimmune diseases, here we directly compared the effectiveness of distinct classes of ROCK inhibitors for their effects on the production of IL-17 and IL-21 by T cells from patients with SLE. Our results demonstrate that inhibition of ROCK-mediated cytokine production could be achieved by multiple therapeutic modalities. In line with prior observations demonstrating that the selective ROCK2 inhibitor, KD025, can downregulate the production of IL-17 and IL-21 by RA T cells, KD025 decreased the production of these cytokines by SLE T

cells, although the production of IFN γ appears to be ROCK2 dependent in RA but not in SLE T cells.¹⁸ The ability of KD025 to target IL-17A and IL-21 production in our study was, however, not accompanied by decreases in Stat3 phosphorylation or IRF4 expression,^{18 36} possibly due to the later timing of addition of the ROCK2 inhibitor to the cultures, which may have enabled the initial wave of signalling events to be protected from the inhibitory effects of KD025.

The increased ROCK activity observed in human Th17 cells as compared with Th0 cells was also effectively decreased by broad ROCK inhibitors such as Y27632 and simvastatin and by the selective ROCK2 inhibitor, KD025, suggesting that the elevated ROCK activity in this T helper subset primarily reflects activation of the ROCK2 rather than the ROCK1 isoform. These results support previous murine and human studies, which have implicated ROCK2 as the major ROCK isoform involved in the differentiation of Th17 cells and T_{FH} cells generated under Th17 cell skewing conditions and have demonstrated beneficial effects of ROCK inhibitors on the production of IL-17 and IL-21 and autoimmune pathogenesis^{9–19 36} and indicate that directly assessing cellular ROCK activity can be helpful in evaluating the efficacy of distinct ROCK inhibitors.

Interestingly, immune gene profiling using NanoString nCounter Technology uncovered broad differences among the distinct classes of ROCK inhibitors. In particular, the transcriptional profile altered by KD025 included inhibitory effects on the expression of the IL-23 and IL-21 receptors suggesting that this compound may target the production of key Th17 cytokines and the cytokine responsiveness of Th17 cells. More studies will, however, be required to determine the functional relevance of these latter effects. In contrast to KD025, the immune profile modulated by Y27632 and simvastatin was largely overlapping. It remains to be determined whether the differences between the selective ROCK2 inhibitor (KD025) and the broader ROCK inhibitors (Y27632 and simvastatin) are due to the ability of the latter two compounds to concomitantly decrease ROCK1 activity and/or are due to additional ROCK-independent actions.

In summary, dysregulated ROCK activity is a consistent feature of PBMCs from autoimmune patients. Targeting of this pathway can be achieved by a number of both selective and non-selective inhibitors suggesting that this pathway could be amenable to a number of distinct therapeutic modalities. Whether ROCK2 selective inhibitors will provide a more advantageous risk/benefit profile than broader ROCK inhibitors will likely require a better understanding of the functional relevance of ROCK1-dependent pathways in immune cells and the development of tools to specifically monitor the activation of ROCK1-specific and/or ROCK2-specific pathways in individual patients.

Contributors ABP was involved in overall study conception and design as well as providing critical input on data interpretation and drafting of the manuscript. JES was also involved in study concept and design, data interpretation and drafting of the manuscript. CR was involved in experimental design, carrying out experiments, data acquisition and analysis, and drafting of the manuscript. YC performed NanoString analysis, contributed to the statistical analysis and was involved in the drafting of the manuscript. RKM recruited patients with RA and HCs, performed PBMC isolations and was involved in data interpretation. SG performed ROCK assays and western blots. LL recruited patients with SLE. KAK provided patients with SLE. VPB and SMG provided critical input on data interpretation. All authors helped to critically revise the intellectual content of the manuscript and approved the final submission.

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