Supporting Information

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SI Materials and Methods

Cell Purification. The whole blood was incubated (20 min, 22 °C) with a RosetteSep human $CD4^+$ T-cell enrichment mixture or a $CD4^+CD127^-$ T-cell enrichment mixture (StemCell Technologies). The remaining unsedimented cells were loaded onto Ficoll-Paque Plus (Amersham Bioscience), isolated by density centrifugation, and washed with PBS. Tregs were isolated from the $CD4^+CD127^-$ population by using the human CD25-positive selection kit from Stem Cell Technologies. Umbilical cord blood (UCB) $CD25^+$ and $CD25^-CD4^+$ T cells were isolated from frozen UCB units (National Placental Blood Program, New York Blood Center) by positive selection using directly conjugated anti-CD25 magnetic microbeads. Cells were cultured with anti-CD3/CD28 mAb-coated Dynabeads for 18–21 d and split every 2–3 d. Recombinant IL-2 (300 IU/mL; Chiron) was added on day 3 and maintained for culture duration. The expanded cells were used for Western blot analysis.

Cell Activation. $CD4^+$ T cells were cultured in complete RPMI 1640 (Invitrogen) containing antibiotics and 10% heat-inactivated FBS. Cells were cultured at a final concentration of 2×10^6 cells/mL (cytokine secretion) or 2×10^5 cells/mL (proliferation) on anti-CD3 mAb (5 μg/mL) and anti-CD28 mAb (5 μg/mL) (eBioscience) precoated 24-well plates. For Th17-skewing activation, IL-1β (50 ng/mL) and TGF-β (5 ng/mL) (R&D Systems Inc.) were added to the culture. Cytokine secretion was determined by ELISA after 48 h by using Human IL-10 and IFN-γ Cytoset (Biosource), IL-17 and IL-2 (R&D Systems Inc.), and IL-21 (eBioscience). Proliferation was assessed by CFSE dilution after 72 h.

Treg Suppression Assay. Human $CD4^+CD127^-CD25^+$ regulatory T cells (Tregs) were purified using the Stem Cell Technologies human regulatory T-cell kit. Tregs were stained with Cell Proliferation Dye eFluor 660 (eBioscience) at 5 μM, and total PBMCs were stained with CFSE (eBioscience) at 5 μM as per the manufacturer's instructions. Tregs were then incubated with KD025 at the indicated concentrations for 5 h before being washed three times in complete RPMI to remove the excess drug. The 3×10^5 Tregs and total PBMCs from the same donor were then placed in culture in a U-bottom 96-well plate with anti-CD3 (OKT3) at a concentration of 1 μg/mL and a final volume of 200 μL. Cells were incubated for 4 d at 37° and 5% CO_2. Cells were then stained with Viability Dye eFluor 780 and CD4 and analyzed by flow cytometry. All FACS plots displayed were gated on $CD4^+$ Viability Dye^- $CFSE^+$ and eFluor 660^- for analysis of only the target CD4 cell population. Percent of inhibition was calculated by comparing activated PBMC alone to those wells in which PBMCs were cultured in the presence of Tregs.

CFSE Labeling. We added CFSE to the cell suspensions ($1–5 \times 10^6$ cells/mL) at a final concentration of 0.5 μM, 37 °C, for 30 min; we stopped the reaction with FCS at a final concentration of 10%. We washed cells twice with PBS and resuspended them with complete RPMI media.

Flow Cytometry. For cytokine intracellular staining, cells were stimulated for 4 h with Cell Stimulation Mixture (00-4975-03; eBioscience) (which contains phorbol 12-myristate 13-acetate, ionomycin, and protein transport inhibitor), fixed, and permeabilized. Cells were stained with antibodies to human or mouse CD4 (Clone RPA-T4 or RM4-5), IL-17 (Clone eBio64DEC17 or eBio17B7), IL-21 (Clone eBio3A3-N2 or FFA21), RORγt (Clone BD2), and IFN-γ (Clone 4S.B3) (eBioscience). Foxp3 expression was tested by using an anti-Foxp3 antibody (Clone 236A/E7) and a staining kit (eBioscience) Samples were analyzed in a guava easyCyte Flow Cytometer machine (EMD Millipore).

Western Blot. Total cell lysates and nuclear and cytoplasmic extracts were prepared and analyzed for protein content. Sample buffer was then added, and after boiling, the samples, containing equal amounts of proteins, were separated on a SDS/PAGE gel and transferred to a nitrocellulose membrane. The membranes were blocked and probed with the specific antibodies overnight. Anti-ROCK1 (HPA007567) and anti-ROCK2 (HPA007459) were purchased from Sigma-Aldrich, and anti-beta actin antibodies (4970S) were purchased from Cell Signaling. Anti-pSTAT1 (#9171), pSTAT3 (#4113), pSTAT4 (#5267), pSTAT5, pSMAD2/3, pSTAT6 (#9361), Lamin B1 (#9087), pMLC (#3674), total MLC (#3672), total STAT3 (#4904), and Tbet (#5214) were purchased from Cell Signaling Technologies. Anti-IRF4 (sc-48338) and anti-RORγt (14-6988) were obtained from Santa Cruz Biotechnology Inc. and eBioscience, respectively. Immunoreactive protein bands were visualized using HRP-conjugated secondary antibodies and an enhanced ECL system.

ChIP Assay. Peripheral blood $CD4^+$ T cells were stimulated by anti-CD3/28 mAbs, IL-1β (50 ng/mL), and TGF-β (5 ng/mL) for 48 h in the absence or presence of 10 μM KD025. Cells were harvested at 48 h, and ChIP assays were performed. For ChIP, cells were cross-linked with 1% formaldehyde for 10 min, quenched with 0.125 M glycine, washed with cold PBS, and lysed in buffer A (50 mM Hepes, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% TX100, 0.1% Na-Deoxycholate, 0.1% SDS, and 1× protease inhibitor). Lysates were sonicated, and after centrifugation, supernatants were incubated overnight at 4 °C with protein G-Dynal beads that preconjugated with desired antibodies. The immunoprecipitates on dynal beads were washed with buffer B (20 mM Tris, pH 8.1, 500 mM NaCl, 2 mM EDTA, 1% TX100, and 0.1% SDS) and then with Tris EDTA (TE) buffer before being eluted with buffer C (1% SDS in TE) at 65 °C for 1 h, and the eluates were then incubated at 65 °C for 6 h or overnight for cross-link reversal before being purified with ChIP DNA clean and concentrator (Zymo Research). Binding of factors were determined by real-time PCR with designed primer sets. IL21-1, IL21 promoter; IL21-2, distal IL21 site was predicted to be high for H3K27Ac. IL17A4, IL17A promoter; IL17A5, distal IL17A site was predicted to be high for H3K27Ac. The control site is an irrelevant site that is not active in CD4+ T cells.

Planar Lipid Bilayers. Planar lipid bilayers containing anti-CD3 antibodies (5 μg/mL) and ICAM-1 (250 molecules/mm^2) were prepared in parallel-plate flow cells as described previously (1). The flow cell containing the bilayers was warmed up to 37 °C, cells were injected in 500 μL of Hepes-buffered saline containing 1% human serum albumin, and images were collected on a custom automated Nikon inverted fluorescence microscope.

Microscopy. All TIRF imaging was performed on the custom automated Nikon inverted fluorescence microscope using the 100×/1.45 N.A. TIRF objective from Nikon. TIRF illumination was set up and aligned according to the manufacturer's instructions as previously described (2). Briefly, cells interacted with the bilayers for 30 min at 37 °C, fixed with 2% PFA, permeabilized with 0.05% Triton-X 100, blocked and stained with

rabbit polyclonal antibodies to ROCK1 and ROCK2 for 20 min, and then incubated with fluorescently tagged goat anti-rabbit Fab_2 (Invitrogen). Controls included the use of nonimmune species-matched IgG. Measurement of signaling was done as previously described (1).

Collagen-Induced Arthritis in Mice. The 8-wk-old male DBA/1J mice were obtained from Jackson Laboratories, and the study was performed in accordance with institutional guidelines and with approval by the Institutional Animal Care and Use Committee of New York University. The mice were immunized via a 0.1-mL intradermal injection at the base of the tail with 100 μg chicken type II collagen (Chondrex, LLC) emulsified with an equal volume of complete Freund's adjuvant containing 4 mg/mL heat-denatured *Mycobacterium* (Chondrex) (day –50). KD025 was administered intraperitoneally to mice with established mild arthritis on day 0 (25 d after immunization; clinical score, 6–8). Mice (n = 10 per group) were dosed intraperitoneally with 50, 100, and 200 mg/kg of KD025 or vehicle (0.4% methylcellulose) once a day for 28 d. Clinical scores were monitored every other day. On day 28, mice were killed and spleens were isolated and analyzed by FACS and Western blot analysis.

Evaluation for Clinical Arthritis. Clinical signs of arthritis were evaluated to determine arthritis incidence. Each paw was evaluated and scored individually using a 0–4 scoring system. The paw scores were summed to yield individual mouse scores, with a maximum score of 16 for each animal. The scoring system was as follows: a paw score of 0, no signs; 1, mild swelling confined to the tarsal bones or ankle joint; 2, mild swelling extending from the ankle to the tarsal bones; 3, moderate swelling extending from the ankle to the metatarsal joints; and 4, severe swelling encompassing the ankle, foot and digits, or ankylosis of the limb.

1. Fooksman DR, et al. (2010) Functional anatomy of T cell activation and synapse formation. *Annu Rev Immunol* 28:79–105.

2. Varma R, Campi G, Yokosuka T, Saito T, Dustin ML (2006) T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. *Immunity* 25(1):117–127.

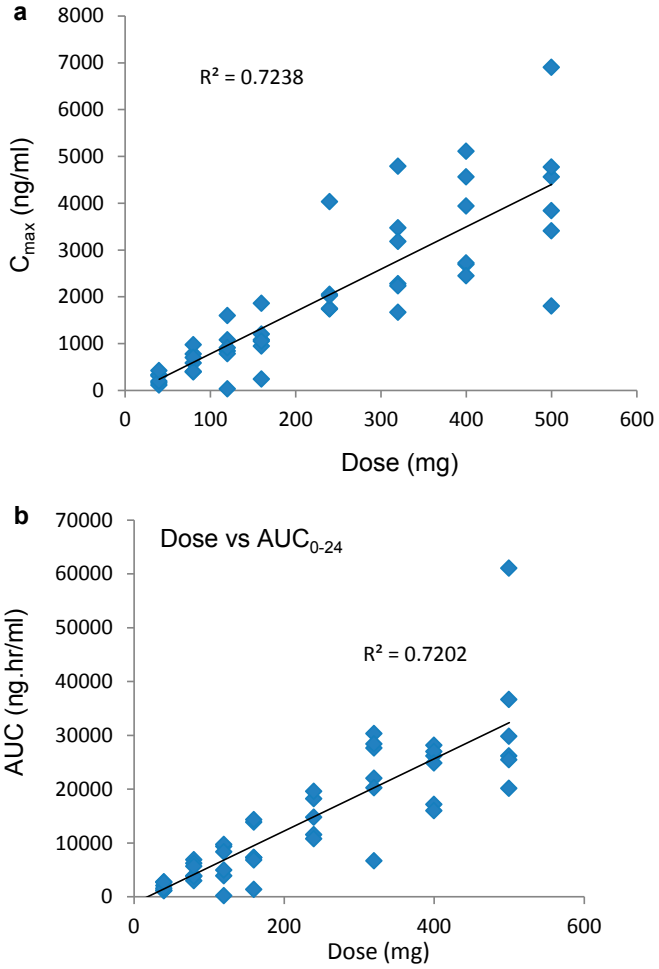

Fig. S1. PK analysis of KD025 in a single ascending dose/multiple ascending dose phase 1 clinical study. The maximum concentration (C_{max}) (*A*) and area under the curve during 24 h ($AUC_{0–24}$) (*B*) of KD025 observed after administration are linear and dose proportional at doses 40–500 mg.

System Organ Class Preferred Term	Placebo N = 16	40 mg N = 6	80 mg N = 6	120 mg N = 6	160 mg N = 6	240 mg N = 6	320 mg N = 6	400 mg N = 6	500 mg N = 6
Gastrointestinal Disorders	0	0	1 (16.7%)	0	0	0	0	0	0
Lip blister	0	0	1 (16.7%)	0	0	0	0	0	0
General Disorders and Administration Site Conditions	2 (12.5%)	0	0	0	1 (16.7%)	0	0	0	0
Fatigue	2 (12.5%)	0	0	0	0	0	0	0	0
Edema peripheral	0	0	0	0	1 (16.7%)	0	0	0	0
Infections and infestations	1 (6.3%)	0	0	0	0	0	1 (16.7%)	1 (16.7%)	0
Furuncle	0	0	0	0	0	0	1 (16.7%)	0	0
Upper respiratory tract infection	1 (6.3%)	0	0	0	0	0	0	1 (16.7%)	0
Injury, Poisoning and Procedural Complications	0	0	0	0	0	0	1 (16.7%)	0	1 (16.7%)
Mouth injury	0	0	0	0	0	0	1 (16.7%)	0	0
Thermal burn	0	0	0	0	0	0	0	0	1 (16.7%)
Investigations	0	0	0	0	0	1 (16.7%)	0	0	0
Blood creatine phosphokinase increased	0	0	0	0	0	1 (16.7%)	0	0	0
Musculoskeletal and Connective Tissue Disorders	2 (12.5%)	0	1 (16.7%)	0	0	0	0	0	0
Back pain	1 (6.3%)	0	1 (16.7%)	0	0	0	0	0	0
Muscular weakness	2 (12.5%)	0	0	0	0	0	0	0	0
Musculoskeletal pain	1 (6.3%)	0	0	0	0	0	0	0	0
Pain in extremity	1 (6.3%)	0	0	0	0	0	0	0	0
Nervous System Disorders	3 (18.8%)	0	1 (16.7%)	0	1 (16.7%)	0	0	0	1 (16.7%)
Dizziness	2 (12.5%)	0	0	0	0	0	0	0	0
Headache	1 (6.3%)	0	1 (16.7%)	0	0	0	0	0	1 (16.7%)
Paraesthesia	0	0	0	0	1 (16.7%)	0	0	0	0
Somnolence	1 (6.3%)	0	0	0	0	0	0	0	0
Respiratory, Thoracic and Mediastinal Disorders	1 (6.3%)	1 (16.7%)	0	0	0	0	0	0	0
Nasal congestion	1 (6.3%)	1 (16.7%)	0	0	0	0	0	0	0
Skin and Subcutaneous Tissue Disorders	1 (6.3%)	1 (16.7%)	0	0	0	0	0	0	0
Dermatitis contact	0	1 (16.7%)	0	0	0	0	0	0	0
Hyperhidrosis	1 (6.3%)	0	0	0	0	0	0	0	0

Fig. S2. Summary of adverse effects observed in a phase 1 clinical trial.

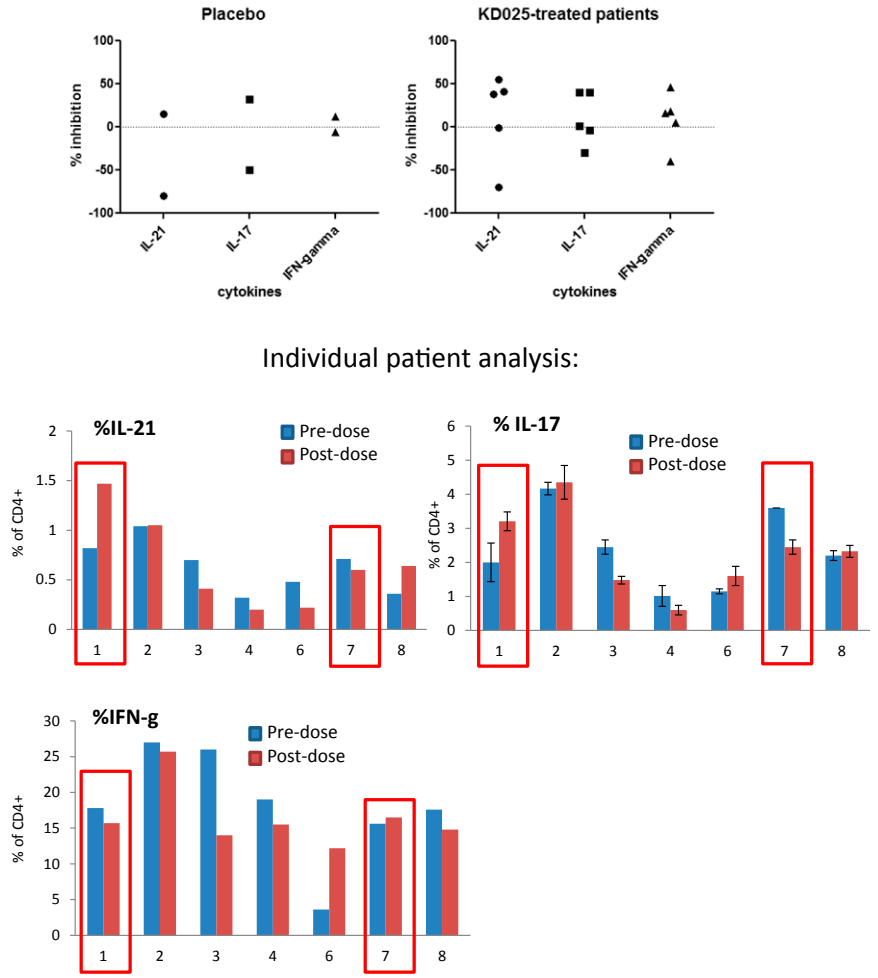

Fig. S3. Oral administration of KD025 has no effect on frequencies of IL-17, IL-21, and IFN-γ–producing $CD4^+$ T cells. Human PBMCs were purified from healthy human subjects before and after oral administration of KD025 (400 mg) and stimulated for 4 h with phorbol 12-myristate 13-acetate and ionomycin, fixed, and permeabilized. Cells were stained with antibodies to CD4, IL-17, IL-21, and IFN-γ and analyzed by flow cytometry. Percent of inhibition was calculated as 1 – (percentage day 15/percentage day 0). Each dot represents the individual patient.

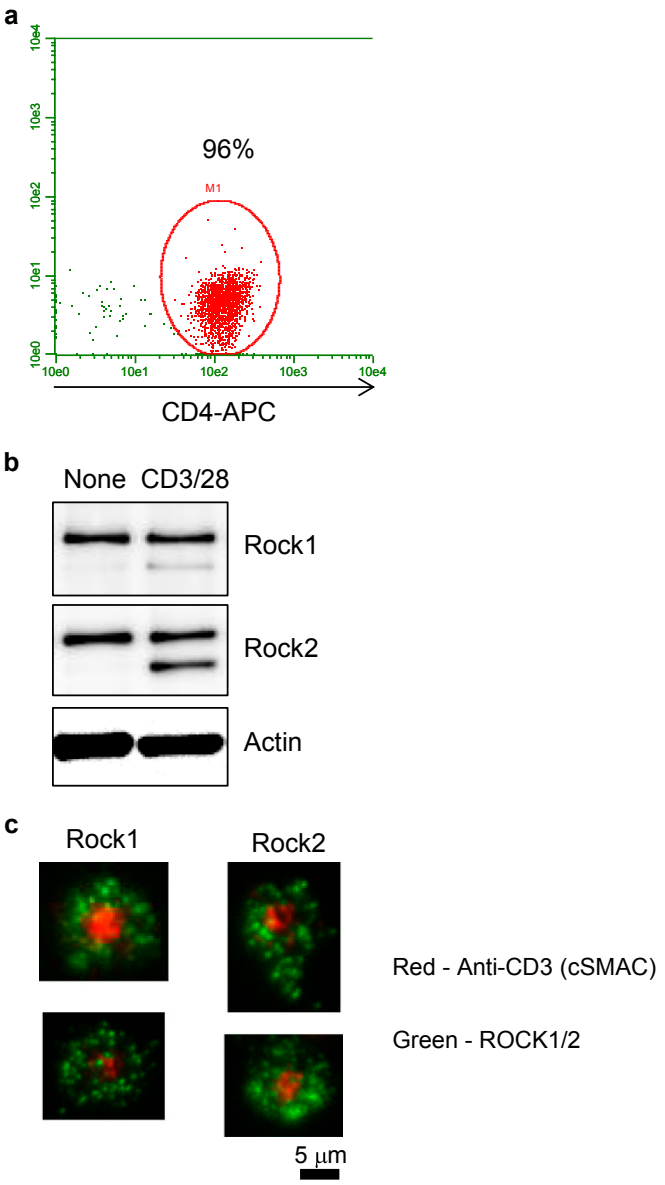

Fig. S4. ROCK1 and ROCK2 are expressed and recruited to the IS in human CD4$^+$ T cells. The percentage of CD4+ T cells was defined by flow cytometry after purification (*A*). Whole cell lysates were prepared from resting or stimulated (by immobilized anti-CD3/28 mAbs) peripheral blood CD4$^+$ T cells and analyzed by Western blot for expression of ROCK1 and ROCK2 (*B*). CD4$^+$ T cells were introduced into bilayers containing anti-CD3 (5 μg/mL) and ICAM-1 at 250 molecules/m^2, fixed at 30 min, permeabilized, stained with anti-ROCK1 or ROCK2 antibodies, and imaged by TIRFM. The panels show representative images (*C*). APC, allophycocyanin.

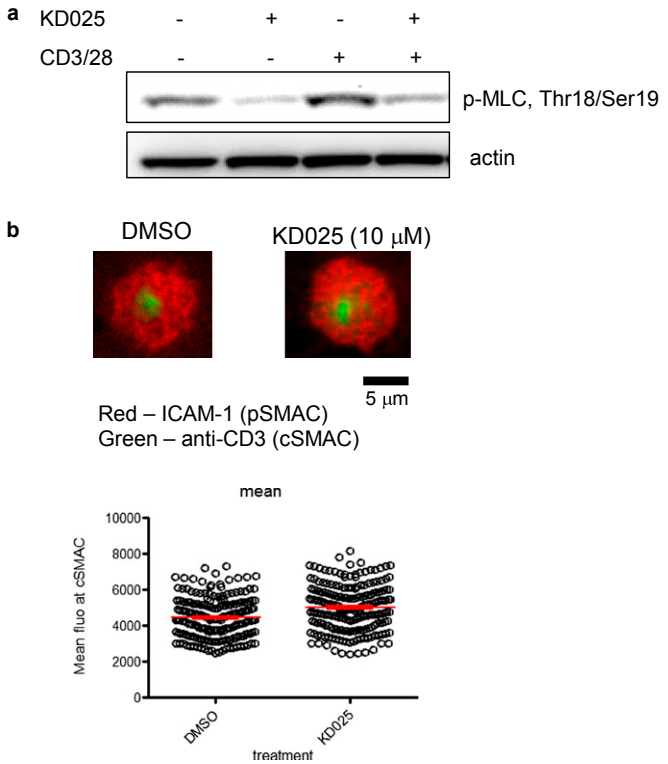

Fig. S5. Selective ROCK2 inhibitor KD025 inhibits phosphorylation of MLC. $CD4^+$ T cells were treated with 10 μM of KD025 for 1 h and then stimulated by immobilized anti-CD3/28 mAbs (*A*) or introduced into bilayers containing anti-CD3 and ICAM-1, fixed, and imaged by TIRF microscopy (*B*). Whole cell lysates were prepared and analyzed by Western blot for phosphorylation of MLC. Actin was used as a loading control (*A*). The panels show representative images (*B*). cSMAC mean fluoresence was quantified in cells (each dot represents an individual cell). One representative experiment of two is shown.

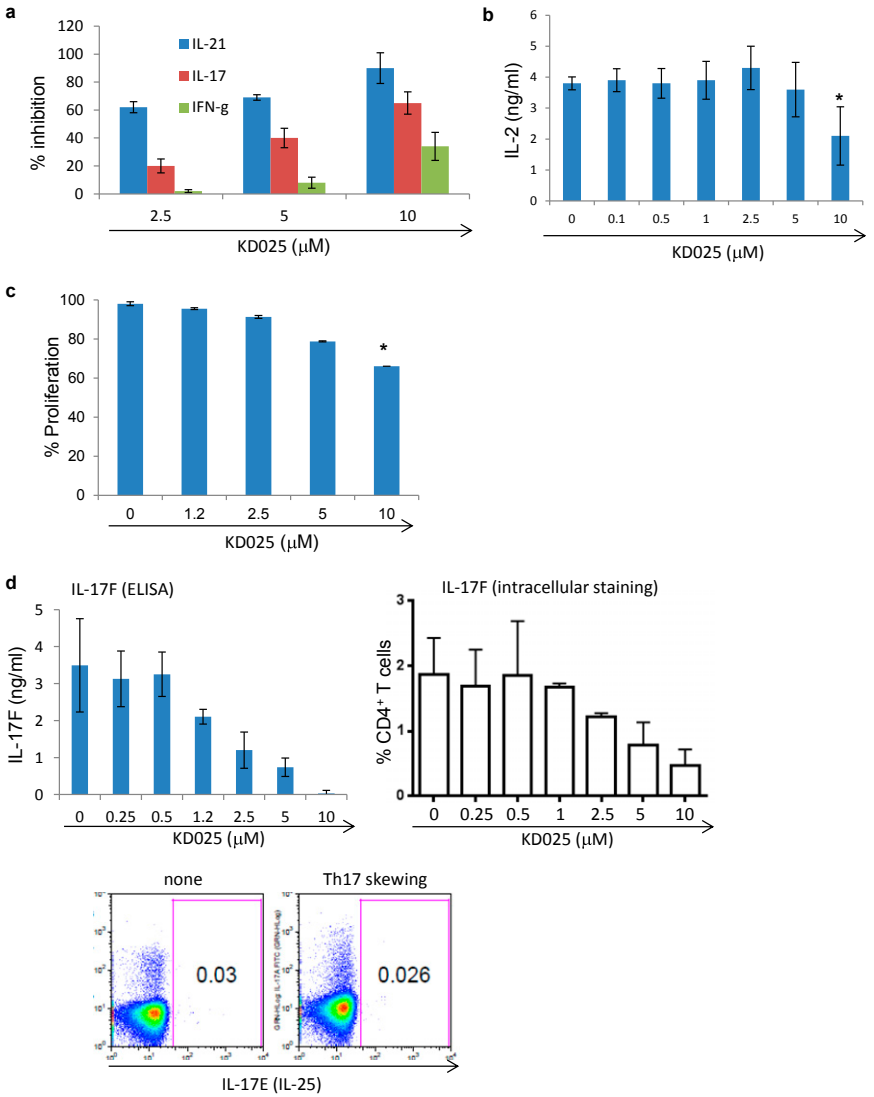

Fig. S6. KD025 inhibits cytokine secretion in previously stimulated $CD4^+$ T cells. $CD4^+$ T cells were stimulated by immobilized anti-CD3/28 mAbs for 5 d, treated with KD025, and then restimulated with anti-CD3/28 mAbs for an additional 48 h. The supernatants were analyzed for IL-21, IL-17, and IFN-γ by ELISA. The data are presented as percent of inhibition, and the average of three different experiments is shown (*A*). $CD4^+$ T cells were treated with KD025 at 0.1 to 10 μM for 1 h and then stimulated by immobilized anti-CD3/28 mAbs in combination with IL-1β (50 ng/mL) and TGF-β (5 ng/mL). IL-2 and IL-17F secretion was analyzed by ELISA after 48 h (*B* and *D*). The average of five (*B*) or three (*D*) different experiments is shown. Proliferation of cells was defined by CFSE dilution after 4 d (*C*). The average of three different experiments is shown. The percentage of IL-17F– and IL-17E–producing T cells was determined by intracellular staining by FACS (*D*).

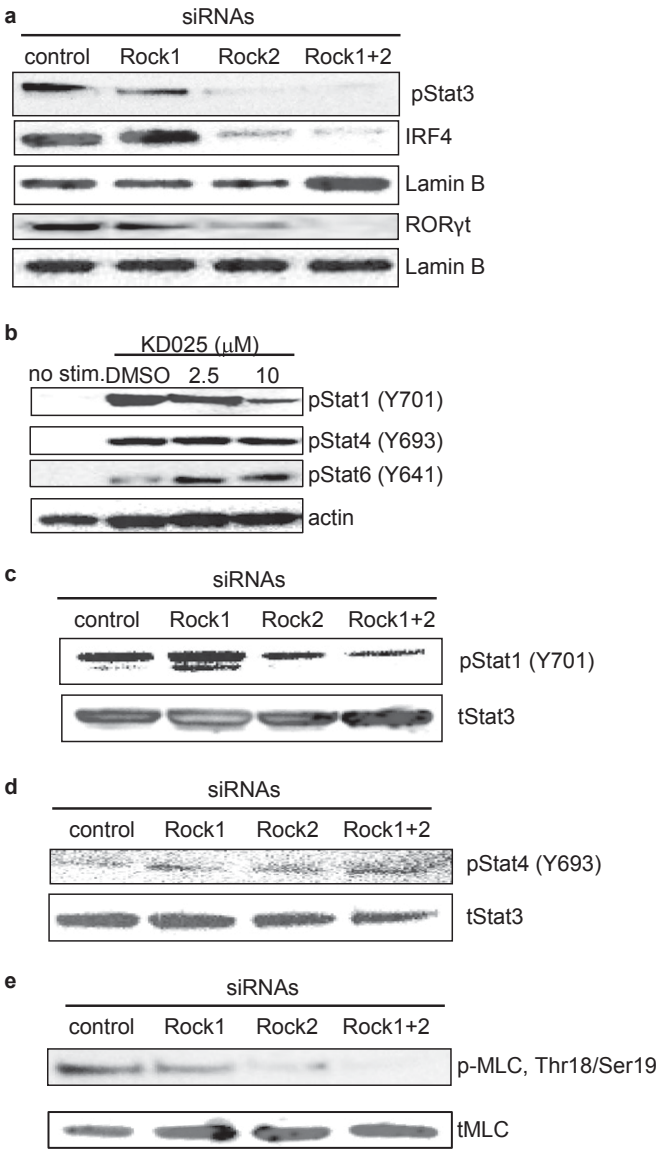

Fig. S7. ROCK2 but not ROCK1 inhibition leads to down-regulation of STAT3 phosphorylation and reduces protein levels of RORγt and IRF4. Peripheral blood $CD4^+$ T cells were transfected with siRNA targeting ROCK1 and ROCK2 or with control siRNA (*A*, *C*–*E*) or treated with KD025 (*B*) and then stimulated by anti-CD3/28 mAbs, IL-1β (50 ng/mL), and TGF-β (5 ng/mL) for 48 h. Whole cell extracts were prepared and analyzed by Western blot by using antibodies against pSTAT3 (*A*), IRF4 (*A*), RORγt (*A*), pSTAT1 (*B* and *C*), pSTAT4 (*B* and *D*), pSTAT6 (*B*), and pMLC (*E*). One representative of three different experiments is shown.

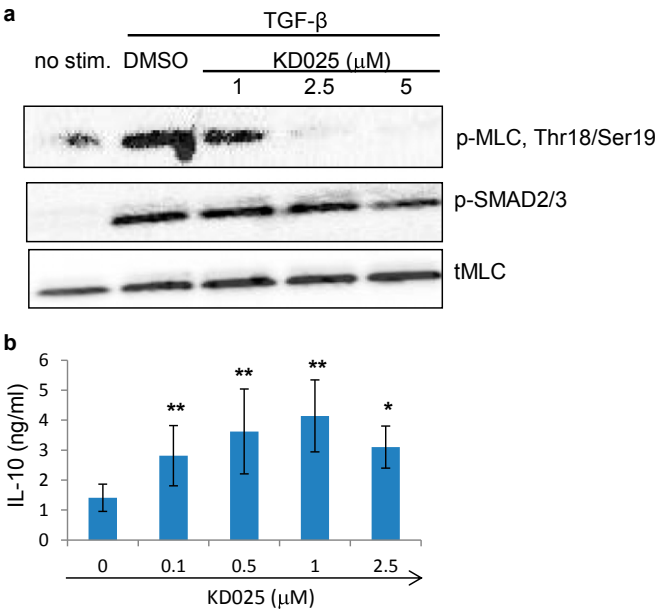

Fig. S8. KD025 down-regulates TGF-β–induced MLC phosphorylation in a SMAD2/3-independent manner. Human $CD4^+$ T cells were treated with KD025 and then activated by TGF-β (5 ng/mL) for 1 h. Whole cell lysates were analyzed by Western blot for MLC phosphorylation. One representative of three different experiments is shown (*A*). IL-10 secretion was analyzed by ELISA after 48 h. The average of five different experiments is shown (*B*).

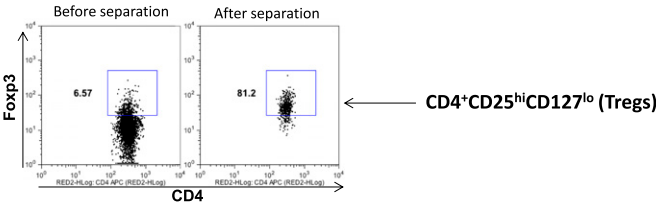

Fig. S9. Purification of human Tregs. $CD4^+CD25^{high}CD127^{lo}$ T cells were purified from healthy donors by positive selection by MACS (about 80% of $Foxp3^+$).

a

sample	age	gender	ethnicity	DAS score
1	64	F	Hispanic	5.98
2	34	F	Hispanic	3.81
3	42	F	Hispanic	2.23
5	35	F	Hispanic	6.15
6	43	M	Hispanic	6.23
7		F	Hispanic	3.19
8	45	F	Hispanic	3.6
9	42	F	Hispanic	6.75
10	32	F	Hispanic	1.9
11	53	F	Hispanic	6.78
12	31	F	Hispanic	2.44

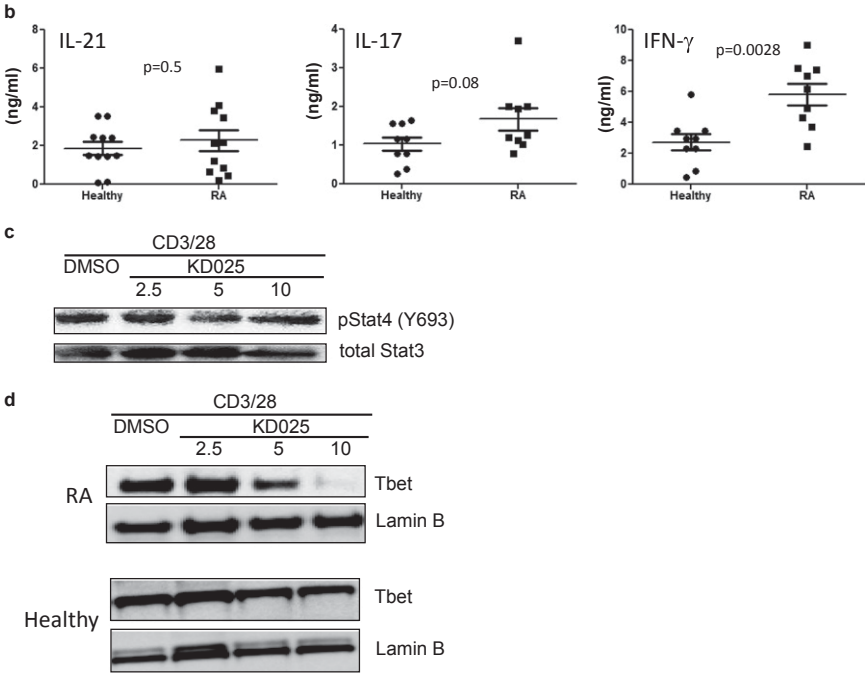

Fig. S10. CD4$^+$ T cells purified from RA patients secrete higher amount of IFN-γ compared with healthy controls upon stimulation ex vivo. The clinical and demographic details of studied RA patients are as follows: DAS, disease activity score; F, female; M, male (*A*). Peripheral blood CD4$^+$ T cells from RA patients were stimulated by immobilized anti-CD3/28 mAbs. IL-21, IL-17, and IFN-γ secretion was analyzed by ELISA after 48 h (*B*). The *P* value was calculated by *t* test. Whole cell lysates were analyzed by Western blot for STAT4 phosphorylation (*C*) and Tbet expression (*D*). One representative of three different experiments is shown (*C* and *D*).

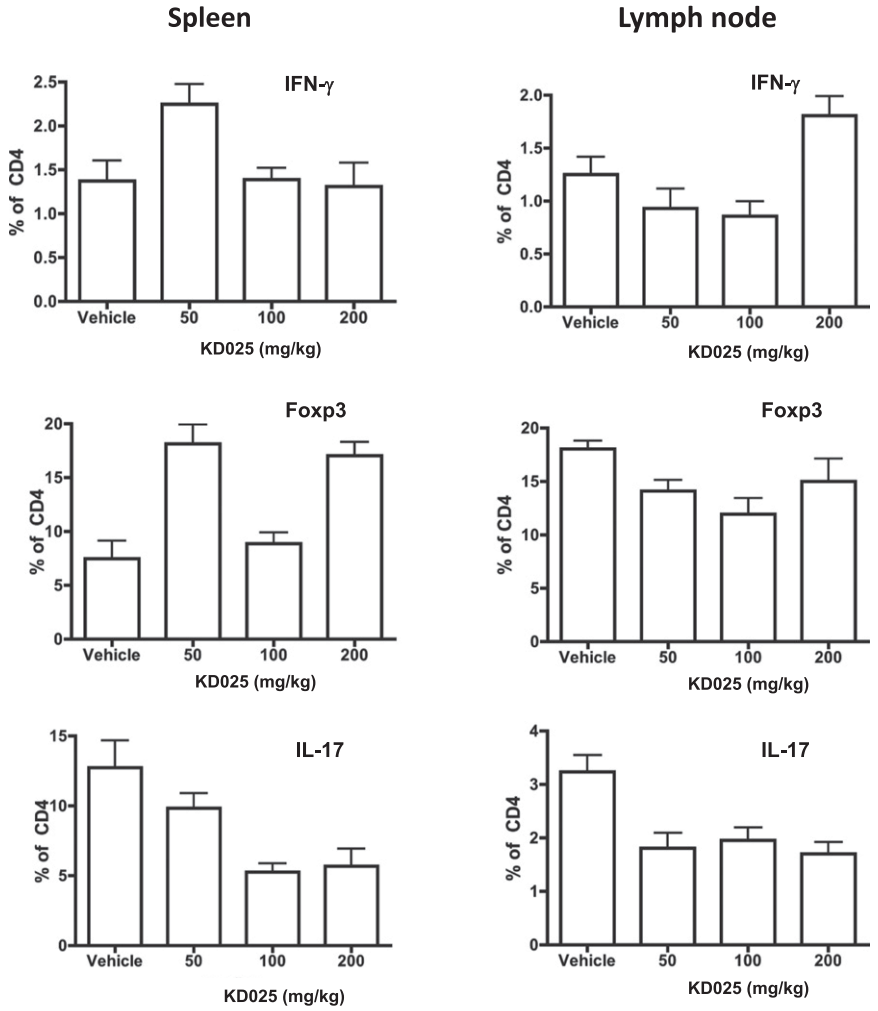

Fig. S11. The effect of KD025 on CD4+ T cells during the progression of CIA in mice. The treatment with indicated doses of KD025 began on day 25 after immunization with collagen. After 28 d, mice were killed, and flow cytometry analysis of spleenocytes and lymph node cells was performed. Data are shown as average percentage (n = 10) ± SD of total $CD4^+$ T cells.

Activity of KD025 against ATP-dependent Kinases and Cell Surface Receptors

Molecular Target	Target Identification	
Kinases[1]	ROCK1, Abl, Akt1, Akt2, AuroraA, Cdk1, Cdk2, CHK1, CK2, c-kit, Erk1, Erk2, p38α, p38β, p38δ, p38γ, DMPK, DRAK, ErbB4, Flt1, Flt4, IKKα, KDR, MAP, MLCK, MRCKα, MRCKβ, MSK1, MSSK1, p70S6K, PDGFα, PHK2, PKA, PKCα, PKGα, PKGβ, PRK1, PRK2, Pyk2, RSK2, Src	
Cell Surface Receptors[2]	Adrenergic: α1, α1, β Dopamine (nonselective) Muscarinic (nonselective) Nicotinic: TTX (+), TTX (-) Norepinephrine Transporter Opioid Serotonergic (nonselective) Na^+ Type 2	Glutamate: AMPA, KA, NMDA Glycine (spinal) Glycine (central) Calcium: Type L, Type N GABA P2X P2Y Potassium: ATP sensitive, Ca^{++} activated, I [Kr]hERG
1 – Inhibition at 10 μM < 40 % to 0 % 2 – No inhibition was observed with KD025		

Fig. S12. Activity of KD025 against ATP-dependent kinases and cell-surface receptors.