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Rho Kinase Promotes Alloimmune Responses by Regulating the Proliferation and Structure of T Cells[1]

Pierre-Louis Tharaux,[2]* Richard C. Bukoski,[†] Paulo N. Rocha,* Steven D. Crowley,* Phillip Ruiz,[‡] Chandra Nataraj,* David N. Howell,* Kozo Kaibuchi,[§] Robert F. Spurney,* and Thomas M. Coffman[3]*

Coordinated rearrangements of the actin-myosin cytoskeleton facilitate early and late events in T cell activation and signal transduction. As many important features of cell shape rearrangement involve small GTP-binding proteins, we examined the contribution of Rho kinase to the functions of mature T cells. Inhibitors of the Rho kinase pathway all had similar actions to inhibit the proliferation of primary lymphocyte cultures. Likewise, transfection of the human Jurkat T cell line with a dominant negative, kinase-defective mutant of Rho kinase diminished Jurkat cell proliferation. Furthermore, inhibition of Rho kinase substantially attenuated the program of cytokine gene expression that characterizes T cell activation, blocked actomyosin polymerization, and prevented aggregation of the TCR/CD3 complex colocalized with lipid rafts. These actions are relevant to immune responses in vivo, as treatment with a Rho kinase inhibitor considerably prolonged the survival of fully allogeneic heart transplants in mice and diminished intragraft expression of cytokine mRNAs. Thus, Rho GTPases acting through Rho kinase play a unique role in T cell activation during cellular immune responses by promoting structural rearrangements that are critical for T cell signaling. ***The Journal of Immunology*****, 2003, 171: 96–105.**

The actin and myosin cytoskeleton appears to regulate T lymphocyte activation at a variety of levels. For example, a number of cell surface proteins involved in T cell function are associated with cytoskeletal complexes, including the ζ-chain of the TCR-CD3 complex (1–3). In addition, the actomyosin cytoskeleton may act as a scaffold for the temporal and spatial distribution of T cell signaling components (4). Small GTPases of the Rho family are key regulators of the cytoskeleton. In nonimmune cells, Rho proteins act as biochemical switches that regulate diverse cellular functions ranging from morphological changes (5–8) and cytokinesis (9) to gene expression and transformation (10). Recent studies have suggested that Rho proteins also have regulatory functions in immune cell populations. For example, Rho-GTPases have been implicated in the regulation of cell shape in a T cell line (11, 12) and in thymocyte homeostasis in transgenic mice (13, 14). In addition, experiments in Jurkat cells have suggested a contribution of Rho in promoting IL-2 production and calcium influx (15). However, a role for these pathways in the regulation of cellular immune responses in primary T cells has not been demonstrated. Moreover, Rho-GTPases exert their biological actions through at least a dozen effectors (16), and characterization of functions for these various effectors of Rho in immune cells is incomplete.

Among the effectors of Rho, Rho kinase (Rho-associated, coiled coil-forming protein kinase (ROCK)[4]) is a major modulator of actin-myosin cross bridge formation through its regulation of myosin phosphatase (17–19) and the myosin light chain (20). Because of the key position of Rho kinase in regulating the actin-myosin cytoskeleton, we studied its role in T cell activation using a panel of inhibitors and by transfection of mutant forms of Rho kinase. Our studies demonstrate a requirement for functional Rho kinase in T cell proliferation, gene expression, and structural rearrangements that characterize T cell activation. These actions contribute to the development of cellular immune responses in vitro and in vivo. Our data identify Rho kinase as a key element of an immunostimulatory pathway that can be targeted pharmacologically to inhibit cellular alloimmune responses.

Materials and Methods

Rho activation assay

Western blot analysis of RhoA was performed as previously described (21) in membrane fractions prepared from mouse splenocytes under control conditions and in the presence of anti-CD3ϵ Ab (1 μg/ml; clone 145-2C11; BD PharMingen, San Diego, CA) or Con A (10 μg/ml; Sigma-Aldrich, St. Louis, MO). Following incubation, primary splenocytes were rapidly frozen in liquid nitrogen, then sonicated in lysis buffer containing 20 mM HEPES-NaOH, 10 mM KCl, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and Complete (Roche, Indianapolis, IN; one tablet per 50 ml). Nuclei and unlysed cells were removed by low speed centrifugation. The supernatant was decanted and centrifuged at 100,000 $\times$ *g* for 30 min to generate membrane and cytosolic fractions. The membrane pellet was then resuspended in the same buffer, and the protein concentration of the fractions was measured and adjusted.

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[4] Abbreviations used in this paper: ROCK, Rho-associated, coiled coil-forming protein kinase; F-actin, filamentous actin; GM1, ganglioside M1; GPCR, G protein-coupled receptor; MLCK, myosin light chain kinase; MLR, mixed lymphocyte response; PH, pleckstrin homology; RB, Rho binding; RPA, RNase protection assay.

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Protein fractions were separated on SDS-15% polyacrylamide gels and transferred to nitrocellulose, and the membranes were probed with a mouse monoclonal anti-RhoA Ab (clone 26C4; Santa Cruz Biotechnology, Santa Cruz, CA). To verify equal loading among the wells, the membranes were also probed with a mouse anti-tubulin mAb (sc-5286). After incubation with HRP-conjugated goat anti-mouse Ab (Amersham Pharmacia Biotech, Arlington Heights, IL), the signal from immunoreactive bands was detected by chemiluminescence using the ECL kit (Amersham Pharmacia Biotech). Western blot analysis was performed twice with protein extracts from four different experiments per condition.

Effects of Rho kinase inhibition on proliferation of splenocytes and T lymphocytes

Single-cell suspensions of splenocytes were prepared from (DBA/2 × BALB/c)F_1 or C57BL/6 male mice as previously described (22). In some experiments pure populations of splenic T cells were isolated using a Pan T Cell Isolation Kit (Miltenyi Biotech, Auburn, CA). The purity of the cell population was confirmed by fluorocytometry and averaged 97%. A range of concentrations of anti-CD3ϵ Ab (up to 300 ng/ml; clone 145-2C11; BD PharMingen) or PMA (6.25 mg/ml) and ionomycin (500 mg/ml) was added to the cells in the presence or the absence of the specific Rho kinase antagonist Y-27632 (3–30 μM; provided by Welfide, Osaka, Japan) (23, 24), the myosin light chain kinase (MLCK) inhibitor ML-7 (2–20 ng/ml; Biomol, Plymouth Meeting, PA) (25), the Rho/Rac inhibitor toxin B from *Clostridium difficile* (0.2–20 ng/ml; Calbiochem, La Jolla, CA) (26, 27), or cytochalasin D (2.5–25 ng/ml; Biomol), which disrupts actin filaments and inhibits actin polymerization (28). After 12, 36, and 60 h of culture, 0.5 μCi of [^{3}H]thymidine was added to each well. Following an additional 12 h of incubation, [^{3}H]thymidine incorporation was assessed by harvesting cells onto a glass-fiber filtermat using an automated Tomtec Cell Harvester (Wallace/PerkinElmer, Palo Alto, CA). Filter-bound radioactivity was measured using a scintillation counter. Each condition was replicated six times in four different experiments.

Transient transfection of Jurkat cells

To examine the functions of Rho kinase in the Jurkat T cell line, cDNAs encoding Myc-tagged constitutively active Rho kinase or full-length or dominant negative forms of Rho kinase were separately cloned into the pEF-BOS-Myc expression plasmids (29, 30). The carboxyl-terminal portion of Rho kinase contains Rho binding (RB) and pleckstrin homology (PH) domains that are autoinhibitory. Binding of the RB domain to Rho induces unfolding of Rho kinase, activating the kinase domain and promoting enzymatic activity. The constitutively active Rho kinase (pEF-BOS-Myc-CA) consists of only the kinase domain of the enzyme, whereas the dominant negative form (pEF-BOS-Myc-RB/PH-TT) contains the RB and PH domains with mutations that were introduced to abolish Rho-activating activity and lacks the kinase domain. Thus, the constitutively active Rho kinase mutant lacking the inhibitory RB and PH domains is permanently active and is not dependent on Rho for activation. By contrast, the dominant negative form of Rho kinase is devoid of any enzymatic activity and has limited interaction with Rho, thereby minimizing interference with other Rho-mediated pathways (28, 29). The plasmids containing the Rho kinase constructs were cotransfected into Jurkat cells along with pGL3-luciferase as an internal control using the X-tremeGENE Q2 kit (Roche) optimized for Jurkat cells according to the recommended procedure. Transfection efficiency averaged ~25%.

Twenty-four hours after transfection, 0.5 μCi of [^{3}H]thymidine was added to each well, and the cells were incubated for an additional 12 h at 37°C in 5% CO_2. Cell proliferation was assessed by [^{3}H]thymidine incorporation as described above. Luciferase activity was measured using the Luciferase Assay System with Reporter Lysis Buffer kit according to the manufacturer's specifications (Promega, Madison, WI). β radioactivity from [^{3}H]thymidine incorporation normalized to pGL3-luciferase activity was measured at least in six different wells for each of the three conditions in two different experiments.

Examination of cytoskeletal morphology

Mouse splenocytes were isolated as previously described (22) and were stimulated for 18 h with anti-CD3 Ab (300 ng/ml) in the presence or the absence of Y-27632 (10 μM). Morphological evaluation was conducted using cytospin preparations after centrifugation at 500 rpm for 5 min. Cells were plated on glass coverslips, fixed with acetone for 5 min, and washed twice with PBS/0.075% saponin and 50 mM NH_4Cl before specific staining of actin filaments with FITC-labeled phalloidin (400 nM; Sigma-Aldrich).

To provide a more quantitative assessment of actin polymerization in lymphocytes, filamentous actin (F-actin) formation was also determined using flow cytometry. To this end, murine splenocytes were harvested and rested for 18 h in RPMI supplemented with 1% FCS. The cells were then stimulated with anti-CD3 (1 μg/ml) in the presence or the absence of Y-27632 (10 μM). The cells were harvested, fixed, and permeabilized with a formaldehyde/saponin solution and stained with an Alexafluor 488-conjugated phalloidin (Molecular Probes, Eugene, OR) that specifically binds F-actin. The cells were then analyzed by flow cytometry, and the percentage of polymerized actin was determined by comparing the percent change in mean channel fluorescence in the activated vs unstimulated cells.

In a different set of experiments performed on cells in suspension, splenocytes were stimulated for 18 h with anti-CD3 Ab (300 ng/ml) or Con A (10 μg/ml), fixed in 3% paraformaldehyde/PBS, and placed between slide and coverslip in aqueous mounting medium. Lipid raft distribution was assessed by staining for the ganglioside M1 (GM1) with FITC-cholera toxin B (0.25 μg/ml; Sigma-Aldrich). Simultaneous staining for CD3 was performed using a rat anti-mouse CD3/TCR complex mAb (clone 17A2; BD PharMingen) and a goat anti-rat Cy3-labeled secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) after FcγR blockade (mouse Fc blocker kit; BD PharMingen) to avoid nonspecific staining of non-T cells by blocking FcγR-mediated binding of Abs to mouse FcγR-bearing cells. Incubation of cells with the same combination of staining agents but with rat anti-CD3/TCR complex mouse monoclonal IgG was performed as a control to ensure that the Cy3-associated red fluorescence was specific to CD3 cellular expression. Specific fluorescence was evaluated with fluorescence microscope (Olympus, Melville, NY) and with a confocal laser scanning microscope (LSM 510; Zeiss, Oberkochen, Germany). The confocal images were captured at 12-bit resolution with an optical slice of 1.2 μm.

Cytokine mRNA expression

Splenocytes were incubated in complete RPMI containing 10% FBS at 37°C. Anti-CD3ϵ mAb (300 ng/ml) or Con A (10 μg/ml) was added to single-cell suspensions of splenocytes with or without the Rho kinase antagonist Y-27632 (10 μM), and cells were harvested after 18 h in triplicate or quadruplicate in two separate experiments. Total RNA was extracted using the TRIzol kit (Life Technologies, Gaithersburg, MD), and cytokine mRNA expression was assessed with a multiprobe template set for mouse cytokines (set mCK1; BD PharMingen) with the RiboQuant RNase protection assay (RPA) system (BD PharMingen). The protected mRNA fragments were quantified with a phosphorimager-based imaging system (Storm 860 PhosphorImager and ImageQuant version 4.2 software; Molecular Dynamics), and the relative expression of each specific band was normalized to the signal for ribosomal L32 mRNA.

An additional experiment was performed to examine the role of Rho kinase in the regulation of cytokine expression in primed cells. Suspensions of splenocytes (60×10^6 total cells) were stimulated with anti-CD3 Ab (1 μg/ml) in T25 flasks. After 48 h, cells were pooled in a 50-ml conical tube, and dead cells were removed on a Ficoll gradient. The remaining live cells were plated on a 12-well plate at a density of 2×10^6 cells/ml and restimulated with anti-CD3 Ab (1 μg/ml) in the presence or the absence of Y-27632 (3 μM). At the same time, naive splenocytes from a wild-type B6 female littermate were plated at the same concentration and stimulated with anti-CD3 Ab (1 μg/ml) in the presence or the absence of Y-27632 (3 μM). After 12 h, RNA was isolated, and RPA was performed as described above.

Mixed lymphocyte response (MLR)

Primary one-way MLR was performed as described previously (31). Suspensions of responder splenocytes were reconstituted at various concentrations and mixed with irradiated stimulator splenocytes at the indicated ratios. After varying periods of incubation (from 6–96 h) with or without Y-27632 (10 μM), cells were pulsed with 0.5 μCi of [^{3}H]thymidine/well for the final 18 h of culture. The cells were harvested, and cell-associated [^{3}H]thymidine content was determined by scintillation counting. Values are expressed as specific counts per minute (counts from wells with responders only subtracted from counts from wells with responders and stimulators), with each point measured in quadruplicate samples in at least three different experiments.

Mouse heart transplantation, histopathological scoring, and intragraft inflammatory cytokine mRNA expression measurement

Heterotopic cardiac transplants in mice were performed as described previously (22). Hearts from C57BL/6 (H-2^b) donor mice were transplanted into MHC-disparate (DBA/2 × BALB/c)F_1 (H-2^d) recipients. All transplant recipients were treated with the specific inhibitor of Rho kinases Y-27632 (23, 24) (20 mg/kg by s.c. continuous infusion with an osmotic pump (Alzet model 2004; Durect, Cupertino, CA; $n = 6$) or isotonic saline

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(0.9% NaCl; $n = 7$)) beginning 2 days before transplant and continuing for 26 days in a survival study. This dose was chosen based on previous studies (32, 33), and the in vivo efficacy of Rho kinase inhibition was further verified in preliminary experiments showing that this regimen prevented the acute pressor actions of infused endothelin-1 (data not shown). Allograft survival was monitored by direct palpation of the heartbeat through the abdominal wall, and graft failure was defined as the cessation of palpable heart beat.

To examine further the effect of the Rho kinase activity on the character of rejection, we evaluated the histopathology of cardiac allografts in the experimental groups. In additional groups of Rho kinase-inhibited ($n = 7$) and vehicle-treated ($n = 7$) animals, cardiac transplants were performed as described above. On day 7 after transplantation, the allografts were removed, fixed in 10% buffered formalin, sectioned, and stained with H&E, and the slides were reviewed by a pathologist (P. Ruiz) who was masked to the treatment groups. The severity of rejection, interstitial infiltrates, myocyte injury, and vascular abnormalities were each graded separately using a semiquantitative scale where 0 was no abnormality, and 1, 2, and 3 represented mild, moderate, and severe abnormalities, respectively (22). Other parts of the allografts were snap-frozen for subsequent RNA extraction and mRNA expression analysis with multiprobes RPA as described above.

Finally, to test whether the Rho kinase inhibitor Y-27632 affected T cell responses in vivo, an additional group of animals was transplanted with heart allografts as described above and treated with vehicle ($n = 4$) or Y-27632 ($n = 4$). Seven days after transplantation, splenocytes were harvested, and one-way MLR was performed as described above. Splenocytes from (BALB/c $\times$ DBA/2)F_1 (H-2^d) recipient mice were stimulated with irradiated splenocytes from the donor strain C57BL/6 (H-2^b) or syngeneic (BALB/c $\times$ DBA/2)F_1 mice. Proliferative responses in MLR were measured after 3 days in culture as described above. Values are expressed as a relative proliferation index. Specific proliferation is determined by subtracting counts in wells with responders only from counts in wells with responders and stimulators. The relative proliferation index is then calculated as a ratio of the specific proliferation with allogeneic vs syngeneic stimulators.

Statistical analysis

The values for each parameter within a group are expressed as the mean $\pm$ SEM. For comparisons between groups, statistical significance was assessed using ANOVA, followed by Tukey's test for multiple comparisons. Differences in survival were evaluated using a log-rank test.

Results

Activation of RhoA in lymphocytes by Con A or anti-CD3 Ab

To determine whether stimulation of T cells is associated with RhoA activation, we measured membrane-associated RhoA in splenocytes before and after stimulation with anti-CD3 Ab or Con A. Activation of RhoA activation is associated with enhanced translocation of RhoA from cytosol to membrane (34). Cross-linking of CD3 with anti-CD3 Ab substantially increased the amount of membrane-associated RhoA by 2.6-fold increase over the untreated control value within 6 h of exposure to the Ab (Fig. 1). This activation of RhoA persisted up to 24 h (3.2-fold over untreated control; $p < 0.05$). Similarly, stimulation of splenocytes with Con A for 24 h also led to a 1.9-fold increase in the amount of RhoA in the membrane fraction (Fig. 1).

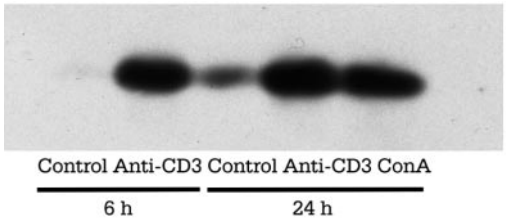


FIGURE 1. Activation of RhoA in lymphocytes by Con A or anti-CD3 Ab. Shown is a representative Western blot of membrane protein fractions from splenocytes stimulated with anti-CD3ϵ mAb (300 ng/ml for 6 and 24 h) and Con A (10 μg/ml for 24 h). The blots were probed with a specific Ab to RhoA. Stimulation with either anti-CD3 mAb or Con A enhances membrane association of RhoA in primary splenocytes compared with quiescent untreated controls ($p < 0.05$ for anti-CD3 or Con A vs unstimulated controls after 24 h; $n = 4$ for each condition in three experiments).

Rho kinase promotes actin polymerization and organization in activated T cells

To determine whether this activation of Rho has functional consequences in T cells and to begin to explore the effector pathways used by Rho in T cells, we initially focused on Rho kinase, a major modulator of actin-myosin cytoskeleton (16). We stimulated mouse splenocytes with anti-CD3 Ab in the presence and the absence of the potent and specific Rho kinase inhibitor, Y-27632 (23, 24). F-actin was barely detectable in quiescent cells (Fig. 2*a*). However, stimulation of mouse T cells with anti-CD3ϵ Ab caused spreading and actin polymerization with formation of a characteristic F-actin ring at the edge of the enlarged, spreading cells (Fig. 2*b*). In contrast, in cells that were incubated with anti-CD3ϵ Ab along with the Rho kinase inhibitor Y-27632, actin polymerization was almost completely inhibited, such that the morphological features of the Y-27635-treated cells were virtually identical with those of the unstimulated, quiescent cells (Fig. 2*c*). However, after 3–18 h of Rho kinase inhibition, pseudopodial extension occurred, as evidenced by cortical F-actin staining in Y-27635-treated cells alone (not shown) or in cells that were incubated with the Rho kinase inhibitor Y-27632 along with anti-CD3ϵ Ab (Fig. 2*c*).

To confirm these effects on actin polymerization, we also measured F-actin formation using a flow cytometry technique. In this assay, exposure of cells to anti-CD3 Ab significantly increased F-actin formation by 19.3 $\pm$ 6.1% compared with untreated controls ($p = 0.004$). By contrast and consistent with the studies shown in Fig. 2, cotreatment with Y-27632 completely inhibited anti-CD3-induced F-actin formation (1.7 $\pm$ 4.7%; $p = 0.03$ vs anti-CD3 stimulation alone; $p =$ NS vs untreated controls).

Rho kinase contributes to lymphocyte proliferation induced by Con A or anti-CD3 Ab

To determine whether the activation of Rho contributes to T cell proliferation, we stimulated mouse splenocytes with Con A in the presence or the absence of Y-27632. Inhibition of Rho kinase with Y-27632 reduced Con A-stimulated splenocyte proliferation in a dose-dependent manner (Fig. 3*a*) up to 70% at maximal doses. The Rho kinase inhibitor also attenuated proliferation when anti-CD3 Ab was the mitogenic stimulus (Fig. 3*b*). In both assays the actions of the Rho kinase inhibitor to attenuate lymphocyte proliferation were more marked at the highest concentrations of mitogen. In data not shown the Rho kinase inhibitor had no effect on baseline proliferation or viability of mouse splenocytes in culture as assessed by trypan blue exclusion and by flow cytometry after staining with anti-annexin V Ab and propidium iodide.

The ability of the Rho kinase inhibitor to reduce proliferation in mixed splenocytes might result from inhibition of Rho kinase in lymphocytes and/or APC. To determine whether Rho kinase activation in T cells modulates the vigor of cellular immune responses, we examined the effects of Y-27632 on proliferative responses of highly purified primary T lymphocytes stimulated by plate-bound anti-CD3 Ab. In vehicle-treated cells, plate-bound anti-CD3 Ab caused vigorous proliferation of purified T cells. Rho kinase inhibition attenuated the proliferative response >50% (95,254 $\pm$ 2,252 vs 41,258 $\pm$ 1,152 specific cpm; $p < 0.0001$; Fig. 3*c*). The Rho kinase inhibitor also caused a modest reduction in the proliferation of purified T cells that were stimulated with PMA and ionomycin (144,232 $\pm$ 3,888 vs 119,397 $\pm$ 1,638 cpm; $p = 0.01$).

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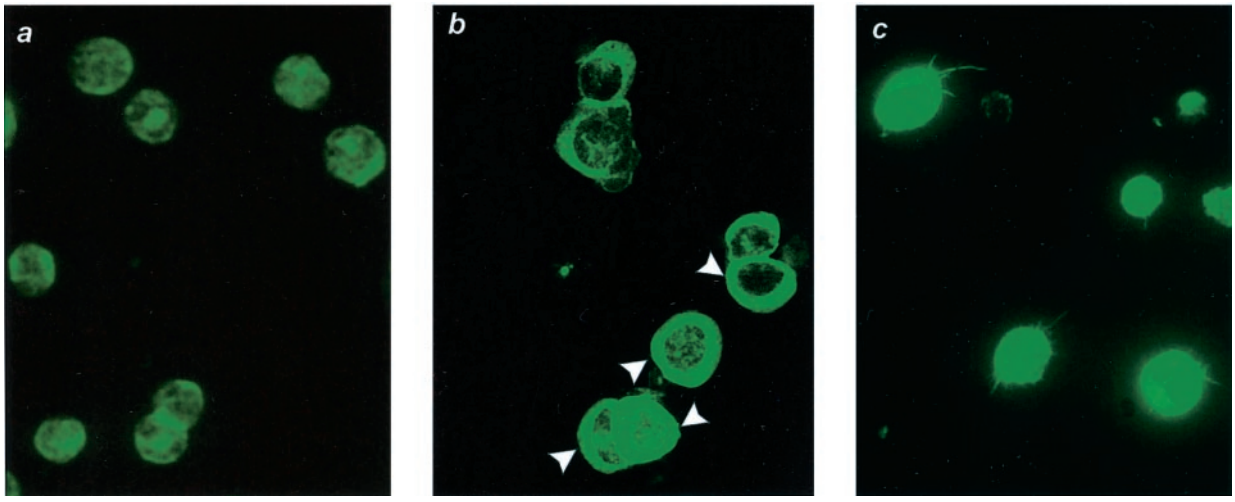


FIGURE 2. Rho kinase promotes actin polymerization and organization in activated T cells. Splenocytes were stained with phalloidin-FITC ($n = 10$ for five experiments). Compared with control unstimulated cells (*a*), cells that were stimulated by anti-CD3ϵ mAb exhibit a bright, thick F-actin cortical ring surrounding the periphery of the nucleus (arrows) after 6 h (*b*). After stimulation with anti-CD3ϵ mAb in the presence of the Rho kinase inhibitor, the F-actin cortical ring is blunted, and pseudopodial extensions are seen, suggesting impaired rearrangement of F-actin filaments (*c*).

Mutant forms of Rho kinase modulate lymphocyte proliferation

To further confirm the biochemical specificity of our results suggesting a role for Rho kinase in T cell proliferation, we next evaluated the actions of transfected mutant forms of Rho kinase on the proliferation of the Jurkat cell line. Jurkat cells were transfected with constitutively active, full-length, or dominant-negative forms of Rho kinase. Sixteen hours after transfection, proliferation was measured. Proliferation was significantly reduced by 50% in the cells transfected with the dominant negative form of Rho kinase compared with those transfected with the constitutively active form of the kinase (Fig. 3*d*). Furthermore, the magnitude of reduction of the proliferative response in Jurkat cells caused by the

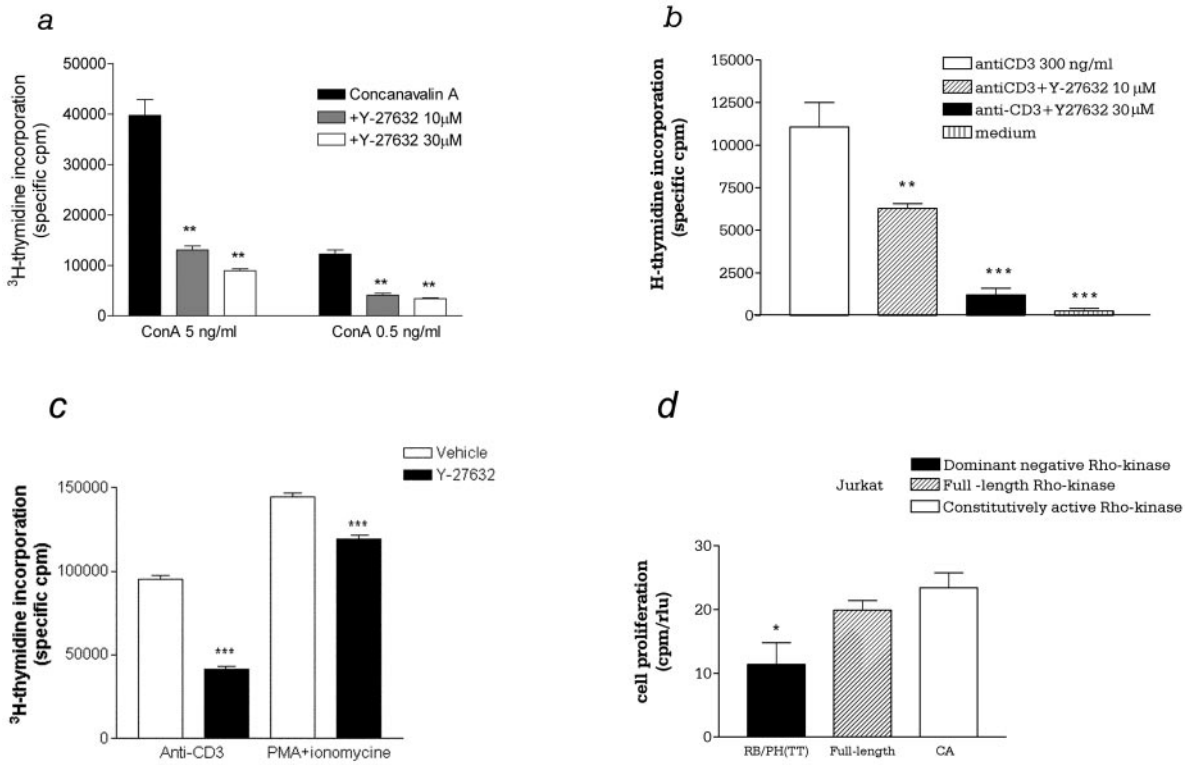


FIGURE 3. Rho kinase promotes T cell proliferation. *a*, Rho kinase inhibition significantly reduced proliferation of Con A-stimulated splenic lymphocytes after 48 h (**, $p < 0.01$ vs Con A alone; $n = 24$ in four experiments). *b*, Pharmacological blockade of Rho kinase impairs anti-CD3-induced splenocyte proliferation after 48 h (***; $p < 0.001$ vs control; **, $p < 0.01$ vs anti-CD3 Ab alone; $n = 24$ in four experiments). *c*, Y-27632 prevents proliferation in purified primary T lymphocytes stimulated with anti-CD3 or anti-CD3 plus PMA and ionomycin (***, $p < 0.001$ vs vehicle; $n = 4$ in each group in three experiments). *d*, Transient expression of a kinase-defective, dominant negative form of Rho kinase reduces the proliferation of Jurkat cells after 36 h (*, $p < 0.05$ vs transfected cells with constitutively active (CA) Rho kinase; $n = 18$ in two experiments). Results are expressed as [^{3}H]thymidine-associated counts per minute per relative luciferase units (rlu) expressed by control pGL3 vector.

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dominant negative construct was virtually identical with that achieved with the pharmacological inhibitor Y-27632 in the same preparation (data not shown).

Blockade of Rho effector pathways upstream and downstream Rho kinase impairs lymphocyte proliferation

To confirm that the actions of Y-27632 on lymphocyte proliferation are due to inhibition of Rho effector pathways that include Rho kinase, we performed additional experiments using a series of agents that interfere with the Rho kinase pathway at steps that are proximal or distal to Rho kinase itself. Because Rho kinase is a distal effector molecule for Rho-GTPases, we tested whether a cell-permeable Rho/Rac inhibitor, toxin B from *C. difficile* (26), could inhibit lymphocyte proliferation. Similar to the effects of Y-27632, toxin B significantly attenuated anti-CD3 Ab-induced proliferation (Fig. 4*a*).

Activation of Rho kinase promotes myosin phosphorylation and the development of actomyosin bridging. Therefore, we next tested whether blocking actin polymerization with cytochalasin D or inhibiting MLCK with ML-7 would affect anti-CD3 Ab-induced T cell proliferation. Similar to the actions of Rho kinase or Rho/Rac inhibition, both compounds substantially inhibited the proliferative response (Fig. 4, *b* and *c*). Moreover, similar to Y-27632, neither agent altered basal proliferation nor adversely affected splenocyte viability (data not shown).

Rho kinase regulates cytokine expression in activated T cells

Activation of T cells induces a characteristic program of cytokine gene expression that shapes the character of the incipient immune response. Stimulation of splenocytes for 18 h with anti-CD3 mAb induces a marked 2-fold increase in IFN-γ and a 4-fold increase in IL-2 mRNA expression (specific signal ratio, 0.97 $\pm$ 0.07 vs 0.43 $\pm$ 0.16 and 0.32 $\pm$ 0.05 vs 0.07 $\pm$ 0.04, respectively; $n = 7$; $p < 0.05$). Enhanced expression of these Th1 cytokines was blunted by concomitant Rho kinase inhibition (specific signal ratio, 0.97 $\pm$ 0.07 vs 0.43 $\pm$ 0.16 and 0.32 $\pm$ 0.05 vs 0.12 $\pm$ 0.01, respectively; $n = 7$; $p < 0.05$; Fig. 5). Expression of the Th2 cytokines IL-4 and IL-13 was also enhanced upon CD3/TCR engagement, and similarly, Rho kinase inhibition attenuated the induction of IL-4 and IL-13 expression by anti-CD3 Ab (0.07 $\pm$ 0.01 vs 0.01 $\pm$ 0.01 and 0.36 $\pm$ 0.11 vs 0.03 $\pm$ 0.01 for IL-4 and IL-13, respectively).

To distinguish whether Rho kinase influences T cell function primarily at the level of activation and expansion or whether it may also modulate effector functions, we examined the effects of the Rho kinase inhibitor on cytokine expression in primed T cells. Splenocytes were first stimulated with anti-CD3 Ab and after 48 h were restimulated with anti-CD3 Ab in the presence or absence of the Rho kinase inhibitor. In the primed cells, anti-CD3 Ab caused a more marked stimulation of cytokine expression than in naive cells (not shown). Whereas enhanced cytokine expression associated with anti-CD3 Ab was attenuated by Rho kinase inhibition in naive cells (Fig. 5), this effect was not observed in primed cells (for example, IL-2, 0.15 $\pm$ 0.05 vs 0.27 $\pm$ 0.06 ($p = 0.19$); IL-4, 0.42 $\pm$ 0.04 vs 0.39 $\pm$ 0.08 ($p = 0.66$)).

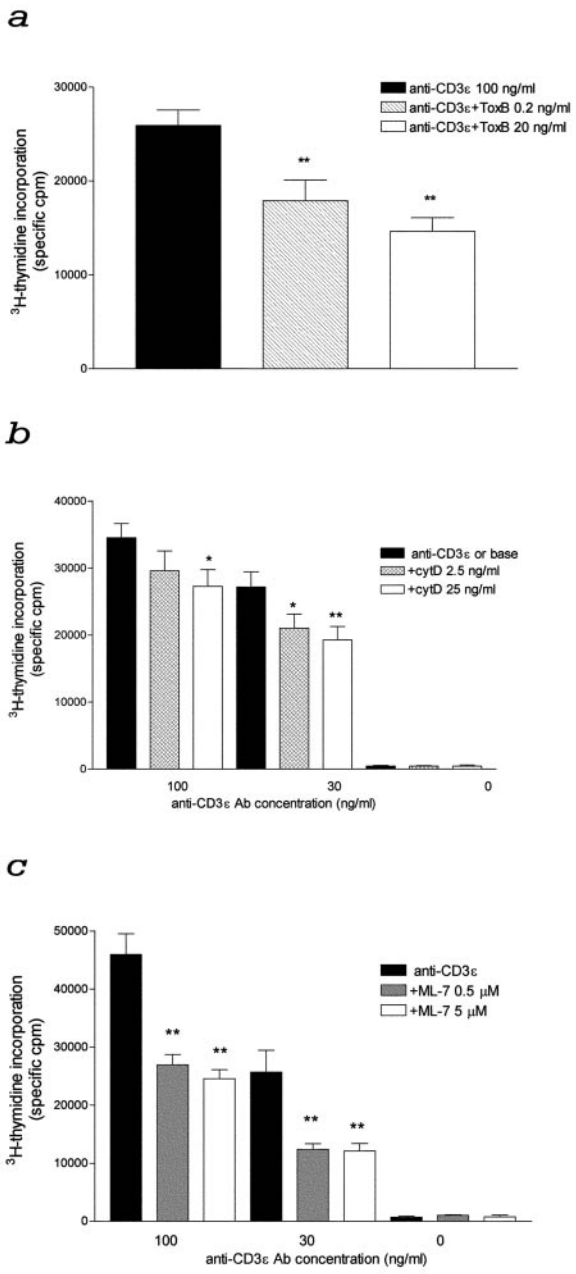


FIGURE 4. Antiproliferative effects of pharmacological blockade of the pathway regulating actomyosin cross-bridging upstream and downstream Rho kinase. *a*, Rho/Rac inhibitor toxin B from *C. difficile* attenuates mouse CD-3-mediated lymphocytes proliferation in a dose-dependent manner. (**, $p < 0.01$ vs anti-CD3 Ab alone; $n = 24$). *b*, [^{3}H]thymidine incorporation is significantly reduced in splenocytes treated with cytochalasin D after anti-CD3 Ab (*, $p < 0.05$; **, $p < 0.01$ (vs anti-CD3 Ab alone); $n = 24$). *c*, ML-7, a specific inhibitor of MLCK, significantly reduces lymphocyte proliferation induced by anti-CD3 Ab (**, $p < 0.01$ vs anti-CD3 Ab alone; $n = 24$).

Rho kinase promotes cellular alloimmune responses

To determine whether Rho kinase contributes to proliferation in a model cellular immune response, we examined alloantigen-induced proliferation in a one-way MLR across a complete MHC mismatch. After 48 h in culture with irradiated, allogeneic (H-2^b) stimulators, proliferative responses by H-2^d responders were significantly blunted by Rho kinase inhibition (Fig. 6*a*). Depending on the responder to stimulator ratio, the Rho kinase inhibitor reduced proliferation by as much as 70% ($p < 0.001$ vs unstimulated conditions; $n = 18$). This was not simply a difference in the kinetics of the response, since proliferation was also reduced by Rho kinase inhibition at 2, 3, and 4 days in MLR (not shown). However, the Rho kinase inhibitor did not affect baseline proliferation or the viability of responder splenocytes. Thus, activation of Rho kinase is required for maximal Ag-specific lymphocyte proliferation in cellular alloimmune responses.

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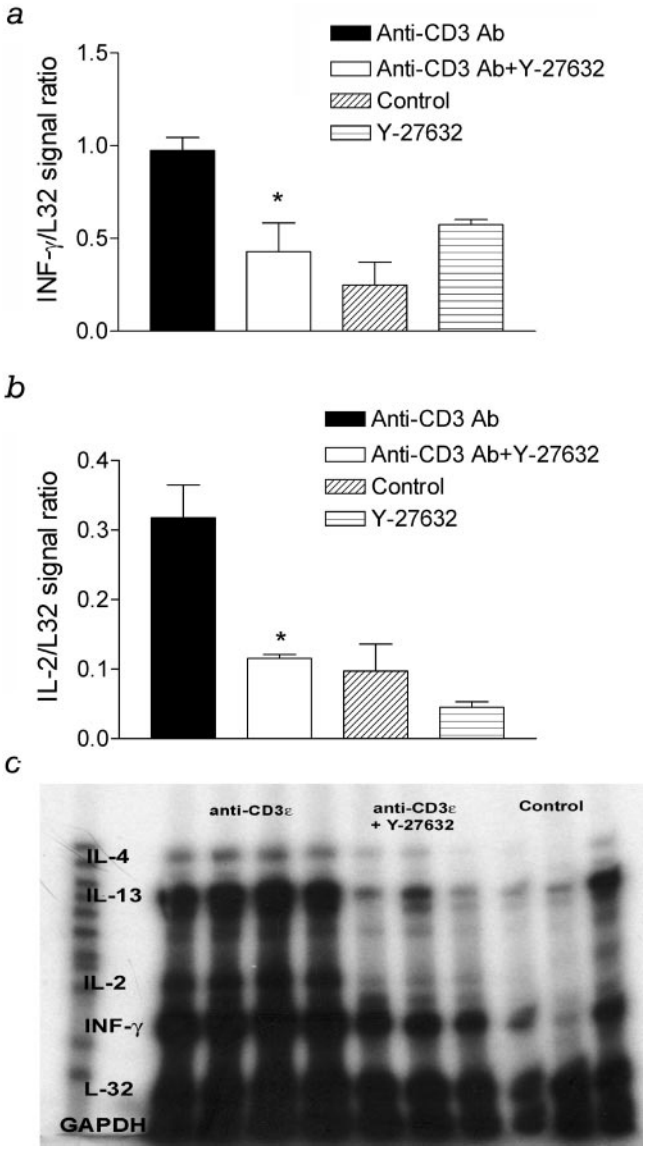


FIGURE 5. Rho kinase regulates cytokine mRNA expression. Cytokine mRNA expression was measured by RPA in splenocytes stimulated with anti-CD3 Ab. Representative phosphorimager analyses show that INF-γ (*a*) and IL-2 (*b*) mRNA expression are abrogated by Y-27632 (10 μM; *, $p < 0.05$ vs anti-CD3ε mAb alone; $n = 6–8$/condition in two experiments). *c*, Representative RPA illustrates global suppression of cytokine mRNA expression by the Rho kinase inhibitor.

A role for Rho kinase in heart transplant rejection

To test whether the actions of Rho kinase in cellular immune responses in vitro are relevant to an immune response in the intact animal, we next assessed the actions of the Rho kinase inhibitor on allograft survival in an aggressive model of cardiac allograft rejection in the mouse. Mice that received MHC-disparate cardiac allografts and vehicle alone all rejected their grafts within 11 days. By contrast, chronic infusion of the Rho kinase inhibitor significantly prolonged allograft survival (Fig. 6*b*). Unlike the controls, none of the Y-27632-treated mice rejected their grafts during the first 2 wk after transplantation, and the mean graft survival was significantly prolonged by administration of the Rho kinase inhibitor (66 ± 33 days) compared with controls that received only vehicle (11 ± 1 days; $p < 0.001$).

To determine the functional consequences of Rho kinase inhibition on the character of rejection, we compared histopathology and cytokine mRNA levels in allografts harvested on day 7 after transplantation. The severity of the overall histopathology was significantly reduced in allografts from animals treated with Rho kinase inhibitor (overall score, 7.2 ± 0.6) compared with the vehicle-treated controls (9.2 ± 0.7; $n = 7$/group; $p < 0.05$; Fig. 6*c*). While there were only modest differences in the intensity and character of the interstitial infiltrates, epicardial injury was significantly reduced ($p < 0.05$). In addition, whereas 43% of controls developed mural thrombi, no mural thrombi were seen in the group that received Rho kinase inhibitor ($p < 0.05$), a finding consistent with reduced severity of allograft injury in the treated group. Despite the relatively modest effects on inflammatory cell infiltration, Rho kinase inhibition markedly inhibited cytokine mRNA expression in the graft ($p < 0.001$ for the global comparison). The expression of IFN-γ and IL-2 mRNA in the allografts was reduced by almost 70% with Rho kinase inhibition (mean specific signal ratio: IFN-γ, 72 ± 12 vs 24 ± 6 ($p < 0.001$); IL-2, 44 ± 6 vs 16 ± 2 ($p < 0.05$); Fig. 6*d*). This pattern of dramatic inhibition of cytokine expression is very similar to that induced by the Rho kinase inhibitor in MLR and suggests that prolongation of graft survival in this setting is due to direct inhibition of T cell activation and effector generation.

Finally, to test whether Rho kinase inhibition affects the development of donor-specific T cell responses in vivo, we examined allospecific proliferation in MLR using splenocytes harvested from mice 7 days after heart transplantation. At a responder-to-stimulator ratio of 2:1, proliferation to donor Ags was reduced significantly (relative proliferation index, 1.66 ± 0.08 vs 0.88 ± 0.05; $p < 0.01$ vs vehicle-treated controls).

Rho kinase regulates lipid raft formation in T cells

To further explore the mechanism of action of the Rho kinase pathway to promote T cell functions, we next examined the effects of Rho kinase inhibition on structural rearrangements in activated T cells. Incubation with Con A caused accumulation of polarized TCR/CD3 complexes in ~30% of splenic lymphocytes (Fig. 7*a*). This pattern was distinct from that in unstimulated, quiescent cells, in which a homogeneous distribution of TCR/CD3 signal was consistently maintained (not shown). Coadministration of Rho kinase inhibitor with Con A prevented the capping of TCR/CD3 complexes (Fig. 7*e*).

Lipid rafts enriched in glycosphingolipids, GPI-anchored proteins, and cholesterol act as functional microdomains in T cell membranes to facilitate activation and signaling. Therefore, we next examined whether Rho kinase activation participates in the formation and trafficking of lipid rafts that are associated with lymphocyte activation. We first tested whether GM1 glycosphingolipids labeled with cholera toxin B subunit were redistributed at the surface of primary T cells after stimulation with Con A. Typically, Con A caused enlargement of TCR/CD3-positive cells and enhanced the density of the FITC-cholera toxin B subunit-associated signal. In 34% of CD3-positive cells, polarization of a lipid rafts marker was observed (Fig. 7*b*), and in this case it was colocalized with TCR/CD3 (Fig. 7, *c* and *d*). Polarization and colocalization of the GM1 marker was never observed when Rho kinase was inhibited ($p < 0.05$ vs Con A-stimulated, CD3-positive cells; Fig. 7, *f–h*). Inhibition of MLCK with ML-7 similarly prevented capping of the TCR/CD3 complex and lipid rafts (not shown).

Discussion

Our studies demonstrate a novel role for Rho kinase to promote T cell activation and proliferation. These actions were identified in primary lymphocyte cultures using a series of chemically distinct

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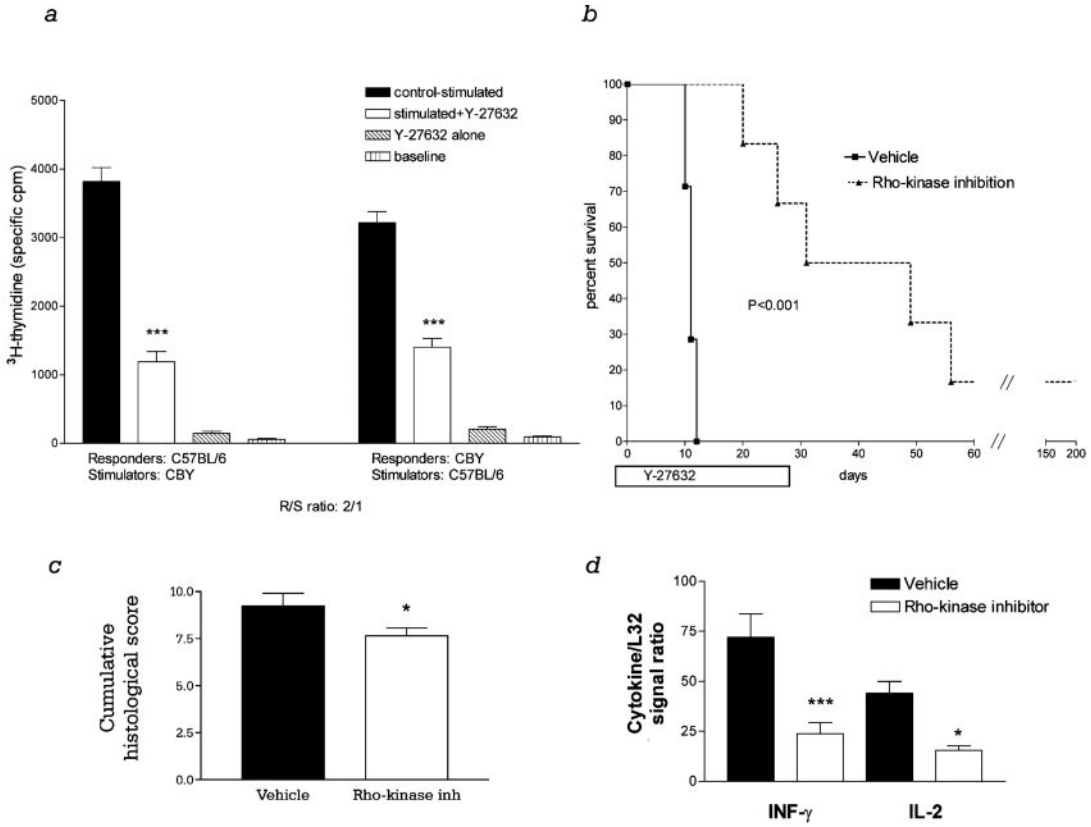


FIGURE 6. Effect of Rho kinase inhibition on the cellular alloimmune response and on cardiac transplant rejection. *a*, Rho kinase inhibition caused a 70% reduction in the proliferative response in a one-way MLR after 48 h (***, $p < 0.001$; $n = 12$ in three experiments). For the combinations tested, this effect was not strain dependent, in that similar inhibition was seen when stimulator and responder strains were reversed (data not shown). *b*, Cardiac transplant survival is substantially prolonged by a short course of treatment with the Rho kinase inhibitor Y-27632 ($p < 0.001$ for survival of Y-27632- vs vehicle-treated group). *c*, The severity of pathological injury was modestly, but significantly, attenuated by Y-27632 ($p < 0.05$). *d*, Intragraft cytokine mRNA levels were reduced by Rho kinase inhibition, as illustrated by the quantitative analysis of INF-γ and IL-2 ($p < 0.001$ and $p < 0.05$, respectively; $n = 7$/group).

inhibitors of the Rho-Rho kinase pathway. Moreover, when Rho kinase was inhibited, there was significant attenuation of lymphocyte proliferation whether activation was induced by Con A, anti-CD3 Ab, or alloantigens provided in the MLR, suggesting that this pathway is required for lymphocyte proliferation in response to diverse stimuli. To confirm the biochemical specificity of this effect, we performed additional experiments using the Jurkat cell line. This lymphoblastic cell line was used because it employs many of the signaling pathways that are present in primary T cells, and it is easily transfected. In the Jurkat line, proliferation was reduced by a dominant negative Rho kinase. The magnitude of inhibition achieved with the dominant negative Rho kinase was similar to that observed with low doses of Y-27632, a compound that specifically inhibits Rho kinase. This observation confirms the specificity of the compound and suggests that its inhibitory actions in this system are due to inhibition of Rho kinase. While the general actions of Rho kinase in Jurkat cells appear to be similar to those in primary lymphocytes, the relative contribution of Rho kinase to regulating proliferation is somewhat less prominent in the Jurkat line. We presume that this reflects properties of cell growth and division that are unique to this transformed cell line.

Previous studies have suggested that Rho kinase activity may modulate certain functions of APCs (35). Thus, it is theoretically possible that some of the effects of Rho kinase inhibition in the anti-CD3-stimulated mixed splenocyte cultures or the MLR experiments might reflect altered APC functions. However, our findings that interruption of Rho kinase signaling inhibits the proliferation of Jurkat cells and purified T lymphocytes stimulated by anti-CD3 Ab indicate a direct effect of Rho kinase in T lymphocytes to promote activation and proliferation. Moreover, the contribution of Rho kinase is more pronounced when proliferation is triggered by TCR engagement, since Y-27632 causes only modest attenuation of T cell proliferation induced by PMA and ionomycin.

The attenuation of proliferative responses by Rho kinase inhibition was associated with a marked reduction of cytokine expression. The suppression of cytokine expression was relatively broad and included marked reductions in the expression of Th1 cytokines such as IL-2 and IFN-γ along with Th2 cytokines including IL-13. These novel findings suggest a key role for Rho kinase in the signaling cascade leading from TCR stimulation to the genomic regulation of cytokine expression. As activation of cytokine gene expression programs is a critical component of T cell activation and proliferation, this marked inhibition of these potent T cell growth factors suggests one mechanism to explain impaired lymphocyte proliferation when Rho kinase is blocked. Our findings are consistent with previous studies showing that ADP ribosylation of Rho by C3 ribosyltransferase inhibits IL-2 production in human T cells isolated from peripheral blood (12) and in Jurkat cells (15). However, the inhibitory effects of C3 exoenzyme may not be completely specific for Rho. Moreover, using this experimental approach, the effector pathways involved in these actions cannot be precisely identified. Accordingly, our study extends previous work by showing that Rho, acting through ROCK/Rho kinase, plays a

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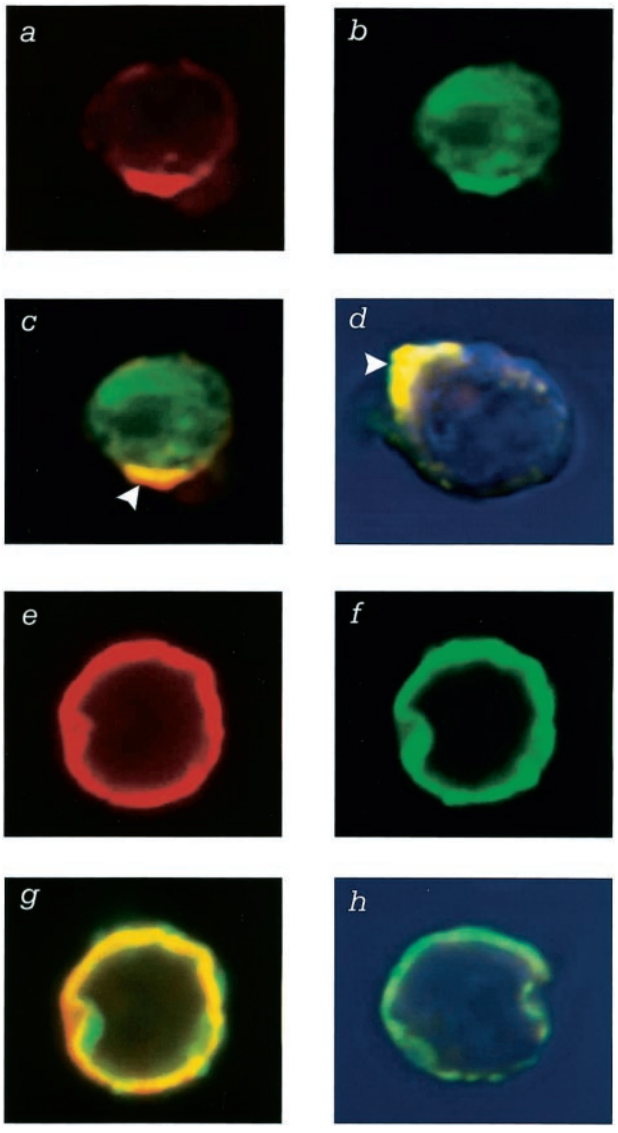


FIGURE 7. Rho kinase regulates lipid raft migration in T cells. Representative confocal photomicrographs showing localization and topographical distribution of TCR/CD3 complex surface Ags (stained red) and lipid raft-associated GM1 glycosphingolipids (stained green). *a*, In the absence of Y-27632, incubation of mouse splenocytes with Con A induced polarization of TCR/CD3 complexes in ~30% of all splenic lymphocytes. *b*, Con A had a similar effect on lipid rafts, as indicated by GM1 glycosphingolipid staining. *c*, As shown in this merged image of *a* and *b*, there was substantial colocalization of TCR/CD3 and lipid rafts in this cell, as indicated by the areas of yellow fluorescence and highlighted by arrows. *d*, As illustrated in another representative lymphocyte, this was observed throughout the specimen. This pattern was distinct from unstimulated, quiescent cells, in which a homogeneous distribution of TCR/CD3 signal was consistently maintained (not shown). *e*, Administration of Rho kinase inhibitor along with Con A prevented the capping of TCR/CD3 complexes. *f*, Redistribution of GM1 glycosphingolipids was also prevented by the Rho kinase inhibitor. *g*, This panel shows merged images of *e* and *f*, again illustrating the inhibition of capping of TCR/CD3 and lipid raft complexes by Y-27632. *h*, This merged image of another $CD3^+$ splenocyte also illustrates the inhibition of polarized accumulation of TCR/CD3 and lipid raft complexes with Rho kinase inhibition. The pattern observed with Rho kinase inhibition is similar to that seen in unstimulated cells.

key role in TCR-mediated stimulation of cytokine gene expression in primary T cells. Furthermore, these actions are critical to Ag-stimulated proliferative responses.

To determine whether these effects of Rho kinase to promote immune responses in vitro were relevant to in vivo immune responses, we examined the effects of Rho kinase inhibition in a model of cardiac allograft rejection. While we were uncertain of the appropriate concentrations of Y-27632 in this circumstance, we selected a previously published dose (32, 33) that was well tolerated and sufficient to inhibit the systemic vasoconstrictor actions of endothelin-1. Using this dose in a model of vigorous cardiac allograft rejection, we found that administration of the Rho kinase inhibitor caused a substantial prolongation of graft survival. Treatment ameliorated some of the histopathological changes of acute rejection, including reduction in epicardial injury and prevention of mural thrombus formation. Although the severity of interstitial inflammatory cell infiltration was not substantially affected, Rho kinase inhibition caused a marked suppression of cytokine mRNA expression in the graft, including IL-2 that is specifically produced by leukocytes. This was very similar to the effects that we observed in cultured lymphocytes and suggests that prolongation of graft survival in this circumstance is primarily due to inhibition of immune cell activation. This interpretation is further supported by the observation that donor-specific alloimmune responses in transplant recipients are attenuated by Rho kinase inhibition.

Our findings confirm a previous study published by Ohki and associates (36) demonstrating improved survival of mouse cardiac allografts using a regimen of more prolonged treatment with Y-27632. However, there were some important differences in the apparent mechanism and impact of Rho kinase inhibition between the two studies. In the study by Ohki et al. (36), treatment with Y-27632 markedly attenuated immune cell infiltration in the graft, and the prolongation of survival was attributed to this impaired leukocyte migration. By contrast, as discussed above, accumulation of immune cells in the graft was not significantly altered in our study despite a similar prolongation of allograft survival. The reason for these discrepancies cannot be readily determined, but strain differences and/or differences in dosage and delivery of the Rho kinase inhibitor may play a role. Nonetheless, our study highlights a role for Rho kinase in rejection that is independent of a major effect on cell migration. In the context of our in vitro data along with the observed diminution of intragraft cytokine expression caused by Y-27632, we suggest that the mechanism of allograft prolongation in this circumstance is due in part to impaired T cell activation and proliferation. These findings indicate that the effects of Rho kinase that we demonstrated in T cells in vitro are relevant to immune responses in the intact animal.

One prominent manifestation of the important linkage between cytoskeletal rearrangements and T cell signaling is the assembly of lipid rafts on the surfaces of activated cells (37–39). These lipid rafts are enriched with proteins that are required for efficient T cell activation (40, 41). The assembly and migration of lipid rafts are key components of the immunological synapse that shapes recognition and activation events. Following TCR ligation, the association of TCR and lipid rafts is dependent on the integrity of the cytoskeleton. Our studies suggest that Rho kinase is required for the normal pattern of TCR and lipid raft migration upon activation of the T cell. We posit that disruption of lipid raft formation is one mechanism that explains the attenuated proliferative response induced by Rho kinase inhibition in T lymphocytes. Our data are consistent with previous work suggesting a role for Rho in altering cell shape in T cell lines (42) as well as studies in cloned NK cells that demonstrated roles for RhoA (43) and ROCK in lipid raft polarization associated with NK cell cytotoxicity (44). Nonetheless, the actions of Rho GTPases and Rho kinase to promote lipid raft formation in primary T cells and an association of this activity

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with T cell activation and proliferation have not been previously documented.

The pathways that lead to Rho-Rho kinase activation in T cells cannot be precisely determined from our studies. However, several possibilities are suggested from previous work. Cytokines such as IL-1 recruit and activate RhoA (45), and an important role for IL-1 in driving proliferation in the MLR is well documented (46, 47). Following G protein-coupled receptor (GPCR) activation, recruitment of Rho GTPases is stimulated by guanine nucleotide exchange factors downstream of G proteins, such as $G\alpha_{12/13}$. A wide array of GPCRs is expressed by T cells, and these receptors modulate T cell functions. For example, chemokine receptors on T cells stimulate chemotaxis and cellular activation. In this regard, Vicente-Manzanares and associates have recently found that ROCK plays a key role in cytoskeletal alterations and chemotaxis induced by the chemokine stromal cell-derived factor 1α (48). Similarly, activation of receptors for thromboxane A2 and angiotensin II promotes cellular immune responses, and these receptors activate Rho GTPases in a variety of nonimmune cells (22, 49). We suggest that Rho kinase activation may be a common pathway used by GPCRs to regulate T cell functions.

Our experiments clearly indicate that inhibition of the Rho kinase signaling pathway has potent effects to impair immune responses in vitro and in vivo. Despite its actions to modulate immune responses, Rho kinase inhibitors appear to be well tolerated in animals. For example, in our study and others (32, 33), chronic administration of Rho kinase inhibitors does not cause significant systemic toxicity. In addition, other beneficial actions of Rho kinase inhibition have been demonstrated in experimental animal models, including reduction of elevated blood pressure (23) and prevention of chronic vascular damage (50–52). Thus, the Rho-Rho kinase pathway, and Rho kinase specifically, are promising therapeutic targets for autoimmune disease and transplant rejection.

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