Analysis of Thymocyte Development Reveals that the GTPase RhoA Is a Positive Regulator of T Cell Receptor Responses In Vivo

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Abstract

Loss of function of the guanine nucleotide binding protein RhoA blocks pre–T cell differentiation and survival indicating that this GTPase is a critical signaling molecule during early thymocyte development. Previous work has shown that the Rho family GTPase Rac-1 can initiate changes in actin dynamics necessary and sufficient for pre–T cell development. The present data now show that Rac-1 actions in pre–T cells require Rho function but that RhoA cannot substitute for Rac-1 and induce the actin cytoskeletal changes necessary for pre–T cell development. Activation of Rho is thus not sufficient to induce pre–T cell differentiation or survival in the absence of the pre–T cell receptor (TCR). The failure of RhoA activation to impact on pre-TCR–mediated signaling was in marked contrast to its actions on T cell responses mediated by the mature TCR α/β complex. Cells expressing active RhoA were thus hyperresponsive in the context of TCR-induced proliferation in vitro and in vivo showed augmented positive selection of thymocytes expressing defined TCR complexes. This reveals that RhoA function is not only important for pre–T cells but also plays a role in determining the fate of mature T cells.

Key words: Rac-1 • Vav-1 • RhoA • antigen receptor • pre–T cell receptor

Introduction

The development and maturation of T lymphocytes in the thymus is an essential process for the formation of the peripheral immune system and is also an exceedingly valuable model for probing the physiological role of signal transduction molecules in vivo. Thymocyte development involves an ordered sequence of differentiation and proliferation. Early thymocyte progenitors, which lack expression of the major histocompatibility receptors CD4 and CD8 ($CD4^-CD8^-$, double negatives [DNs][*]), initiate rearrangements of the TCR-β locus and, if successful, this allows surface expression of a functional pre-TCR complex (1, 2). The pre-TCR instructs cells to proliferate rapidly and to undergo further differentiation to express CD4 and CD8.

$CD4^+CD8^+$ double positive (DP) thymocytes rearrange their TCR-α locus, express a mature TCR α/β complex, and are subjected to the processes of positive and negative selection that generate $CD4^+$ and $CD8^+$ single positive (SP) thymocytes (3, 4).

There has been considerable interest in the signal transduction molecules that mediate antigen receptor signaling in pre–T cells and for thymocyte selection. In particular, Rho family guanine nucleotide binding proteins/GTPases appear to regulate these critical decision points in thymocyte development. This was first noted in mice lacking Vav-1, a guanine nucleotide exchange factor (GEF) with selectivity for Racs (5). Vav-1 loss in lymphocytes causes defects in activation-dependent actin polymerization (6, 7) and a block in pre–T cell differentiation and positive and negative selection (8–10). The action of Vav-1 on the actin cytoskeleton reflects the GEF actions of Vav-1 on Rac-1, a GTPase which selectively organizes filamentous actin structures. The developmental defects caused by loss of Vav-1 in pre–T cells can thus be corrected by expression of an activated mutant of Rac-1 which modulates actin dynamics (11). In this context, activation of Rac-1 potentiates pre–T cell differentiation and proliferation and is

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[*]*Abbreviations used in this paper:* CFSE, carboxyfluorescein diacetate succinimidyl ester; DN, double negative; DP, double positive; GEF, guanine nucleotide exchange factor; LCR, locus control region; NLC, normal littermate control; PdBu, phorbol-12,13 dibutyrate; Rag, recombinase activating gene; SP, single positive.

able to substitute for the pre-TCR complex to initiate T cell differentiation into DPs in recombinase activating gene (Rag)-1 null mice that lack expression of the pre-TCR (11).

The function of a second GTPase Rho is also required for thymocyte development at the pre-T cell stage (12–15). In cell lines, in vitro RhoA can regulate actin cytoskeletal structure, gene transcription, and cell transformation (for a review, see reference 16). However the ability of Rho to control specific cell fate development in vivo has only been explored in the context of thymocyte development. Thus loss of Rho function blocks pre-T cell differentiation and proliferation (13, 14). Rho function is also absolutely necessary for survival of signaling pathways in early thymocyte progenitors (13, 15). The signal transduction pathways used by the pre-TCR to control T cell proliferation and differentiation involve protein tyrosine kinases (17, 18), and Rho has been shown to act downstream of the Src kinase p56lck and to be required for activated p56lck mutants to drive pre-T cell differentiation (19). Rho is also a critical component of the signaling pathways used to suppress the proapoptotic p53 checkpoint during thymocyte development (15).

Despite the overwhelming evidence that Rho function is essential for thymocyte development, it is not known if Rho signaling is sufficient for thymocyte survival, differentiation, and proliferation. One way to probe Rho function further is to examine the effects of gain of function mutants of Rho in the thymus. There have been several studies probing the consequences of expressing active RhoA mutants in leukemic T cell lines. This approach has indicated a role for RhoA in the modulation of T cell polarization and migration (20), in the regulation of T cell spreading after TCR engagement (21), as well as in the potentiation of the transcriptional activity of AP-1 during T cell activation (22). The relevance of these observations to the in vitro role of RhoA in the thymus is not known and results obtained in studies of transformed cell lines must always be subjected to the proviso that they may not mimic what happens under physiological conditions. Accordingly, the objective of this study was to study the consequences of RhoA activation in primary thymocytes and peripheral T lymphocytes. The human CD2 promoter and locus control region (CD2-LCR) was used to express an active mutant of RhoA, V14RhoA in normal T cells of transgenic mice. Analysis of these mice showed that RhoA activation is sufficient to stimulate integrin-mediated cell adhesion in thymocytes but is not sufficient for pre-T cell survival, differentiation, and proliferation. It was also seen that RhoA can strikingly potentiate TCR-α/β–mediated responses in primary lymphocytes both in vitro and in vivo, a previously unrecognized function for this GTPase. This study also reveals crosstalk between the GTPases Rac-1 and Rho in thymocyte development. Activation of Rac-1 is sufficient to induce pre-T cell differentiation whereas RhoA activation is not. Nevertheless, the actions of Rac-1 in the thymus are dependent on Rho signaling.

Materials and Methods

Transgenic Mice. A 0.7-kb cDNA fragment comprising myc epitope-tagged sequences of V14RhoA was cloned in the Sma I site of a transgenic expression vector containing the promoter, the first intron and the LCR of the human CD2 gene (23). The resulting plasmid was cut with restriction enzymes at a KpnI–NotI site to generate 12-kb fragments comprising the promoter and intron sequences and the LCR driving the expression of V14RhoA. These fragments were used to generate transgenic mouse lines following standard protocols. Transgene-carrying mice were identified by PCR of genomic DNA purified from mouse ear punches. The primers used were Vah1 (5′-GGTG-CAGTCTCCAAAGAATTC-3′) and U46 (5′-CCGAAGATC-CTTCTTATTCCCAAC-3′) or Vah4 (5′-GGGGACAAT-GAGTTTTCTGCTG-3′) and RO1 (5′-GTGGGACACAGC-TGGGCAGG-3′). Stable V14RhoA transgenic lines were established by backcrossing transgene carrying founder mice with C57/BL6/J mice. L61 Rac-1 and CD2 C3 transgenic mice, and Vav-1$^{-/-}$ and Rag-1$^{-/-}$ mice have been described in detail elsewhere (10, 11, 14, 24). Mice were bred and maintained under specific pathogen free conditions in the Imperial Cancer Research Fund Biological Resources Unit.

Cell Preparation. Thymi were obtained by dissection from 6–9-wk-old mice. Tissue was disaggregated by mincing with fine forceps and forced through a fine mesh filter to obtain a single cell suspension. Total cell numbers were determined by microscopic observation of Trypan's blue–stained cells using a Neubauer hemocytometer.

Western Blot Analysis. Freshly prepared thymocytes were washed twice in ice-cold PBS and subjected to lysis using buffer, 20 mM Tris, pH 7.5, 10% glycerol, 1% NP40, 150 mM NaCl, 10 mM MgCl$_2$, 1 mM sodium orthovanadate, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml chemostatin, and 1 mM PMSF. The extract was centrifuged at 15,000 g for 15 min at 4°C. Proteins were concentrated by precipitation with 1.5 vol of acetone. Proteins corresponding to 10^7 cells were separated in 15% SDS-PAGE, transferred to polyvinylidene difluoride membranes and detected by Western blot analysis using specific anti-RhoA (26C4) monoclonal antibody (Santa Cruz Biotechnology) and enhanced chemiluminescence (Amersham Pharmacia Biotech).

Flow Cytometric Analysis. Antibodies (BD PharMingen) were obtained conjugated to either FITC, PE, allophycocyanin, or biotin. Biotinylated antibodies were revealed using streptavidin-Tricolor (Caltag). Thymocytes and splenocytes were stained for surface expression of the following markers using the antibodies given in parenthesis: CD8 (53-5.8); CD4 (RM4-5); CD25 (IL-2R α-chain, 3C7); CD3ε (145-2C11); TCR-β (β-chain, H57-597); B220 (CD45R, RA3-6B2); CD44 (Pgp-1, IM7); Thy 1.2 (CD90.2, 30-H12); pan NK DX5; Mac-1 (CD11b, M1/70); Gr-1 (Ly-6G, RB6-8C5); HY-TCR (T3-.70); Vb11-TCR (RR3.15); and Vβ8–TCR (F23.1). Cells were stained with saturating concentrations of antibody at 4°C for 30 min at 10^6 cells per sample, in a 96-well V-bottomed shaped microtiter well plate in 100 μl of PBS containing 1% BSA. Cells were washed with this buffer in between incubations and before analysis on a calibur fluorescence-activated cell sorter (FACS®; Becton Dickinson). Events were collected and stored ungated in list mode using CELLQuest™ software. Live cells were gated according to their forward-scatter and side-scatter profiles and data were analyzed using CELLQuest™ software.

Proliferation Assays. Freshly isolated thymocytes from normal littermate control (NLC) and V14RhoA mice were seeded in

triplicates at 10^6 cells per milliliter in 96 well flat-bottomed microtitre plates (Nunc) in 200 μl of medium RPMI, 10% FCS, and 50 μM 2-β mercaptoethanol. Cells were either left unstimulated or stimulated for 48 h with indicated concentrations of anti-CD3 mAb (145.2C11, coated on plate overnight at 4°C), or with phorbol-12,13 dibutyrate (PdBu) and calcium ionophore ionomycine (Calbiochem) at indicated concentrations. Cells were then labeled with 1 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech), incubated for further 12–18 h, and harvested on filters. Incorporated radioactivity was quantified using a microplate scintillation counter.

Carboxyfluorescein Diacetate Succinimidyl Ester Labeling. Freshly isolated thymocytes were resuspended at a concentration of 10^7 cells per milliliter in RPMI 1640 and labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) by incubation for 5 min at 37°C. After incubation, excess CFSE was quenched by adding excess amounts of FCS and washing in RPMI 1640. For CFSE analysis of cell division, CFSE-labeled thymocytes were seeded in 24–well plates (2 × 10^6 cells per well in RPMI 1640 containing 10% FCS) either uncoated or precoated with anti-CD3 antibody. Cells in uncoated wells were either left untreated or stimulated with Pdbu and ionomycin at the concentrations indicated. After culturing for 48 h, cells were collected and analyzed by flow cytometry for CD4 and CD8 staining and CFSE fluorescence.

Cell Attachment Assays. Flat-bottomed Maxisorp 96-well plates (Nunc) were left uncoated as controls or precoated with different concentrations of mouse fibronectin (Chemicon) in PBS overnight at 4°C. Wells were washed twice with PBS, and nonspecific binding sites were blocked with 1% denatured BSA for 1 h at 37°C. Freshly isolated thymocytes were washed in RPMI and labeled with 2.5 mM BCECF-AM (Calbiochem) in the same medium for 30 min at 37°C, followed by one further wash. Labeled thymocytes were resuspended in RPMI and added to fibronectin precoated plates (10^6 cells per well in 100 ml of medium). Plates were centrifuged at 40 g for 1 min and subsequently incubated for 30 min at 37°C. Nonadherent cells were removed by washing three times in warm RPMI medium. Adhesion was quantified by recording emission at 530 nm, after excitation at 485 nm, using Fluoroscan II (Labsystems, Inc.). Specific adhesion is obtained by subtracting background adhesion (mean reading for wells uncoated with fibronectin) from the reading for each well. This specific adhesion is expressed as a percentage of the total emission before the incubation, i.e., percentage of total cell input adhering to the well.

Microscopy. Freshly isolated thymocytes were plated on 24-well plates (2 × 10^6 cells per well in RPMI 1640) containing 1-cm glass coverslips precoated with either fibronectin at 50 mg/ml or poly-L-lysine (Sigma-Aldrich). After incubation for 1 h at 37°C nonattached cells were removed, the coverslips were washed twice with RPMI 1640, and attached cells were fixed with 4% paraformaldehyde in PBS by incubation for 15 min at room temperature. After fixation, coverslips were washed three times with PBS and mounted upside down to a glass slide using Gelvatol (Monsanto Chemicals). Phase contrast images were obtained with a Zeiss Laser Scanning Microscope LSM 510 with a 63× NA/1.4 planapochromat oil immersion lens (Nikon).

Intracellular Calcium Analysis. Thymocytes were incubated Indo-1 acetoxy-methyl ester (1 mM indo-1; Calbiochem) for 45 min at 37°C in RPMI 1640, 10% FCS, washed, and stained with anti-CD8 tricolor, anti-CD4-PE, and anti-CD3 (145-2C11). Four-color flow cytometric analysis was performed on a BD LSR flow cytometer (Becton Dickinson). Tricolor and PE were

excited by an argon laser (488 nM, 20 mW) and Indo-1 by a UV helium/cadmium laser (325 nM, 8 mW). Indo-1 emission was detected using 424/440 nm (violet) and 510 (blue) bandpass filters. To determine the relative intracellular calcium concentration, cells were warmed to 37°C, analyzed for 1 min to establish baseline calcium levels, and CD3 was crosslinked by the addition of the indicated concentrations of goat anti–hamster IgG (Jackson ImmunoResearch Laboratories). Acquisition was continued in real time for up to 8 min.

Results

Thymocyte Development in Transgenic Mice Expressing Constitutively Active V14RhoA. The human CD2-LCR (23) was used to express a constitutively active mutant of Rho, V14RhoA, in T cells of transgenic mice (Fig. 1 A). V14RhoA expression was determined by immunoblotting of thymocyte lysates with RhoA mAb. V14RhoA is myc epitope-tagged and has reduced migration in SDS-PAGE and can be clearly distinguished from endogenous RhoA. Two V14RhoA transgene-carrying founder lines were established that showed similar levels of V14RhoA expression in the thymus (Fig. 1 B) and similar phenotypes. Thymocyte numbers in V14RhoA thymi were normal (Fig. 1 C) and all major subsets of CD4CD8 DNs, DPs, and SPs were present (Fig. 1 D). There is a huge body of work showing that RhoA regulates changes in the actin cytoskeleton in epithelial cells and fibroblasts (16). The actin cytoskeleton has an important role in regulating cell adhesion. Accordingly, we examined the effect of V14RhoA in thymocyte adhesion; it has been reported RhoA can regulate cell adhesion in lymphocytes (25). Thymocytes express the integrin $\alpha 4\beta 1$ which acts as the main receptor for the extracellular matrix protein fibronectin in these cells (26, 27). We analyzed the attachment of thymocytes from normal and V14RhoA mice to plate-bound mouse fibronectin. The data in Fig. 2 A show that thymocytes expressing active RhoA have higher levels of adhesion to fibronectin than normal thymocytes. The gross morphology of thymocytes expressing V14Rho was indistinguishable from normal adhesive cells (Fig. 2 B).

Characterization of the Effects of Active Rho on TCR-β Selection. A main objective of this study was to examine the effects of Rho activation in pre-T cells because it is known that Rho signals are essential for this stage of thymocyte differentiation (13, 14). CD4$^-$CD8$^-$ DN cells can be subdivided according to two different cell surface markers: CD25 and CD44. The first T cell progenitors are CD44$^+$CD25$^-$ (DN1), followed by the CD44$^+$CD25$^+$ (DN2) stage where TCR-β rearrangements initiate. β rearrangements are completed in CD44$^-$CD25$^+$ (DN3) cells and, if successful, allow expression of functional pre-TCR complexes which instruct cells to proliferate rapidly, down-regulate CD25, upregulate CD2 and CD5, and differentiate into CD44$^-$CD25$^-$ DN4 cells and then to progress to the DP stage. The data in Fig. 3 show CD25 and CD44 staining profiles of DN thymocytes and reveal that V14RhoA thymi have a normal distribution of DN3 and
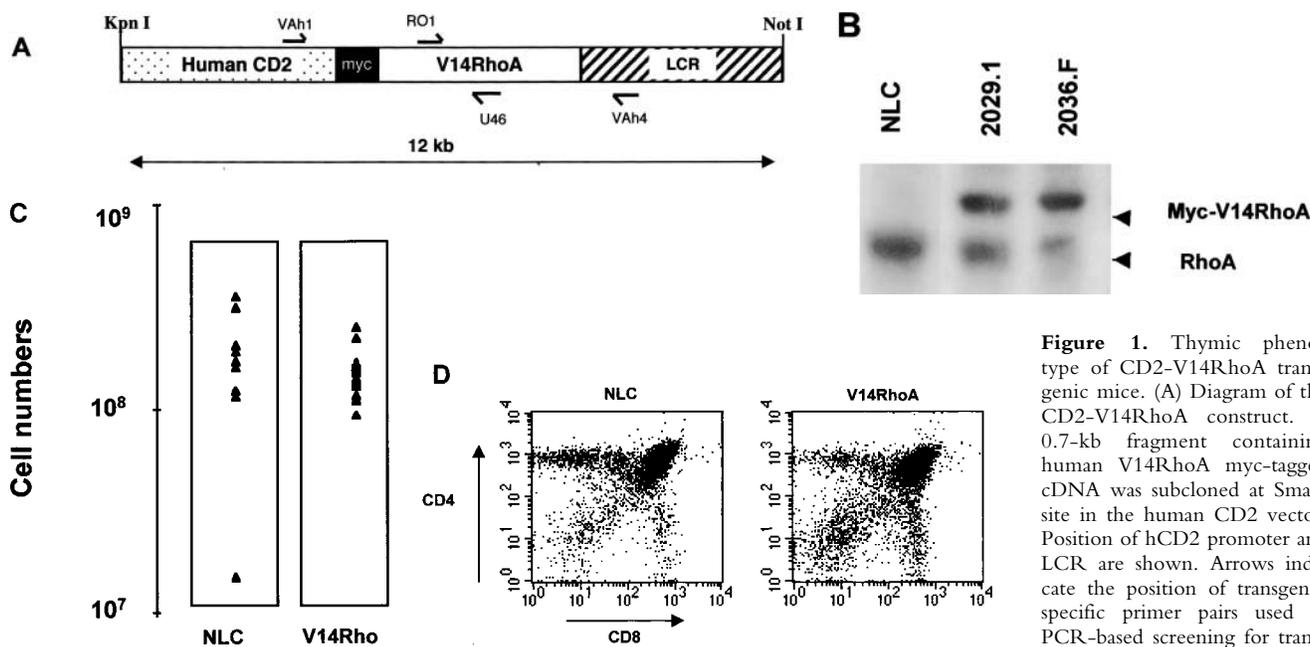
Figure 1. Thymic phenotype of CD2-V14RhoA transgenic mice. (A) Diagram of the CD2-V14RhoA construct. A 0.7-kb fragment containing human V14RhoA myc-tagged cDNA was subcloned at Sma I site in the human CD2 vector. Position of hCD2 promoter and LCR are shown. Arrows indicate the position of transgene-specific primer pairs used in PCR-based screening for transgene-carrying mice. (B) Thymic expression of V14RhoA transgene. Proteins of thymocytes lysates from NLC mice and transgenic mice (V14RhoA) (10^6 cells) were separated on a 15% SDS-PAGE. Detection of endogenous RhoA and transgene V14RhoA was performed by Western blot analysis using a specific monoclonal anti-RhoA antibody. Results are shown for the two different transgenic lines generated (2029.1 and 2036.F). Top arrowhead, myc-tagged transgenic V14RhoA; bottom arrowhead, endogenous RhoA. (C) Absolute thymocyte number in CD2-V14RhoA mice. Thymocyte cell numbers from 6–8-wk-old NLC ($n = 8$) and V14RhoA are shown ($n = 10$). (D) CD4/CD8 profiles on V14RhoA thymocytes. Thymocytes from NLC and V14RhoA mice were isolated, stained with anti-CD4-PE and anti-CD8-FITC, and analyzed by flow cytometry. Two dimensional dot plots representative of five independent experiments are shown.

DN4 cells (Fig. 3 A) and express normal levels of CD2, a marker of pre-TCR function (data not shown). Thus, by these criteria, activation of RhoA has no discernible impact on pre-T cell biology.

To probe the actions of RhoA in more detail, we examined the effects of RhoA activation on thymocyte development in a model of defective pre-T cell differentiation, namely Rag-1 null mice. These mice are defective in anti-
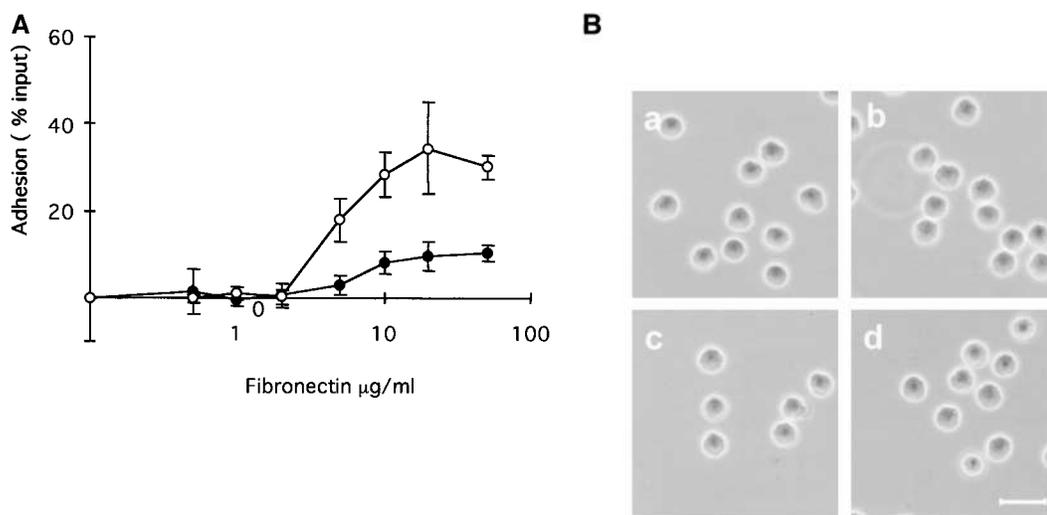

Figure 2. Adhesion to fibronectin is enhanced in thymocytes expressing constitutively active RhoA. (A) Thymocyte adhesion to fibronectin. Cell adhesion of freshly isolated thymocytes to increasing concentrations of mouse fibronectin was assayed as described in Materials and Methods. Specific adhesion is expressed as the percentage of attached cells for NLC (black) and CD2 V14RhoA (white) thymocytes. Data represent means ± SD of triplicates. One experiment representative of three is shown. (B) Cell morphology of thymocytes attached to fibronectin. Freshly isolated thymocytes were attached to fibronectin or poly-L-lysin coated coverslips and analyzed by light microscopy as described previously. Panels show phase contrast images of thymocytes from nontransgenic mice (a and c) or V14RhoA transgenic mice (b and d), attached to fibronectin (a and b) or poly-L-lysin (c and d). Bar, 10 μm.
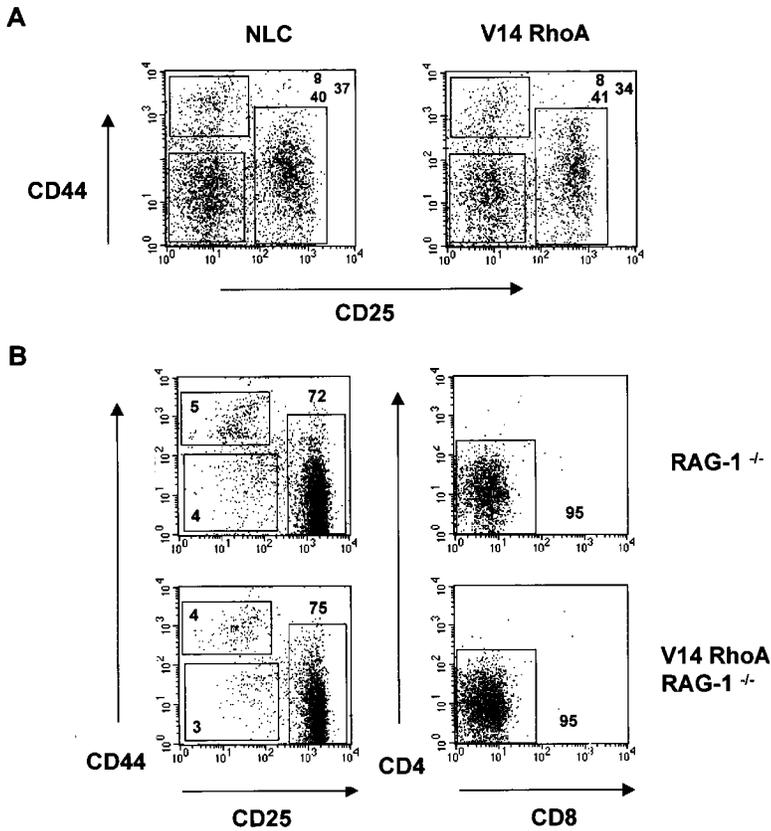
A

B

Figure 3. Effect of activated RhoA on pre-T cell differentiation. (A) Analysis of CD4$^-$8$^-$ DN populations in V14RhoA transgenic mice. Thymocytes from V14RhoA and NLC mice were analyzed for expression of CD25 and CD44 by lineage exclusion of all CD4 and CD8 DP and SP as well as cells of non-T cell lineage using a panel of biotinylated antibodies to CD4, CD8, CD3, B220, Mac-1, NK, γδ, and Gr-1, revealed with streptavidin allophycocyanin, and costained with anti-CD44 CyChrome, anti-CD25 FITC, and Thy-1.2 PE. Dot plots show CD44/CD25 profiles in lineage-, Thy-1.2$^+$ gated cells. Number indicates percentage of total DN thymocytes. (B) Activated RhoA does not restore CD4$^+$CD8$^+$ DP differentiation in Rag-1$^{-/-}$ mice. Thymocytes from Rag-1$^{-/-}$ and V14RhoA/Rag-1$^{-/-}$ were stained for CD25 and CD44 (left panel) as described in A, for CD4 and CD8 (right panel) and analyzed by flow cytometry. Dot plots show CD44/CD25 profiles in Lineage-, Thy-1.2$^+$ cells (left) and CD4/CD8 profiles in Thy-1.2$^+$ cells (right). Results are representative of at least three independent experiments.

gen receptor gene rearrangements and show a block in thymocyte development at the CD4CD8 DN stage because of failed pre-TCR expression (24). It is possible to overcome the lack of pre-TCR in Rag-1$^{-/-}$ mice by expression of gain of function mutants of the Rho family GTPase Rac-1 (11). This GTPase mimics the signaling function of the pre-TCR and induces CD4CD8 DP differentiation when expressed in Rag-1$^{-/-}$ pre-T cells (11). To determine whether expression of active RhoA could induce T cell development in the absence of the pre-TCR complex, V14RhoA transgenic mice were crossed with Rag-1$^{-/-}$ mice. V14RhoA/Rag-1$^{-/-}$ thymi were then examined for the presence of the various thymocyte subsets (Fig. 3 B). In Rag-1$^{-/-}$ mice, thymocyte development is blocked at DN3 stage and CD4$^+$CD8$^+$ cells are not generated. Expression of active RhoA does not change this phenotype (Fig. 3 B). Thymocyte cellularity was low in Rag-1$^{-/-}$ mice and was not rescued by expression of active RhoA.

Activation of RhoA Potentiates TCR-α/β–induced Proliferative Responses In Vitro and In Vivo. TCR levels of DPs were normal in V14RhoA transgenic mice but it was observed reproducibly that SPs that developed in these mice had lower levels of TCR complexes (Fig. 4 A). Therefore, we examined the responsiveness of these cells to activation via the TCR complex with anti-CD3 antibodies. Accordingly, the proliferative responses of thymocytes to plated CD3 antibodies were monitored. Interestingly, despite the lower levels of the TCR complexes on SPs from

V14RhoA mice, these cells were hyperresponsive to plated CD3 antibodies for the induction of proliferation (Fig. 4 B). There was no detectable difference in the response of the cells to activation with the pharmacological reagents calcium ionophore and phorbol ester (Fig. 4 B), demonstrating the specificity of hyperresponsiveness of these cells to TCR/CD3 triggering.

The fluorescent cell permeable dye CFSE can be used to monitor cell division. The data in Fig. 4 C show the CFSE staining profiles of T cells from normal and V14RhoA thymi both before and after activation. Unstimulated NLC and V14RhoA T cells show a single peak of CFSE staining whereas cells activated with CD3 antibodies or calcium ionophore and phorbol esters show multiple peaks of CFSE staining. A higher percentage of V14RhoA T cells (82%) committed to proliferate compared with the NLCs (52%). Moreover, more of the V14RhoA cells had undergone multiple divisions when compared with control cells. In contrast, there was no difference in the CFSE staining profiles of T cells activated with ionomycin and Pdbu.

To probe the mechanisms of RhoA effects on T cells we looked at immediate biochemical responses triggered by the TCR. Ligation of antigen receptors activates cellular tyrosine kinases that, via a network of adapters and enzymes, regulate intracellular calcium. The calcium responses of V14RhoA T cells triggered by CD3 antibodies were indistinguishable from normal cells. The ability of V14RhoA to potentiate T cell responses thus occurs at a
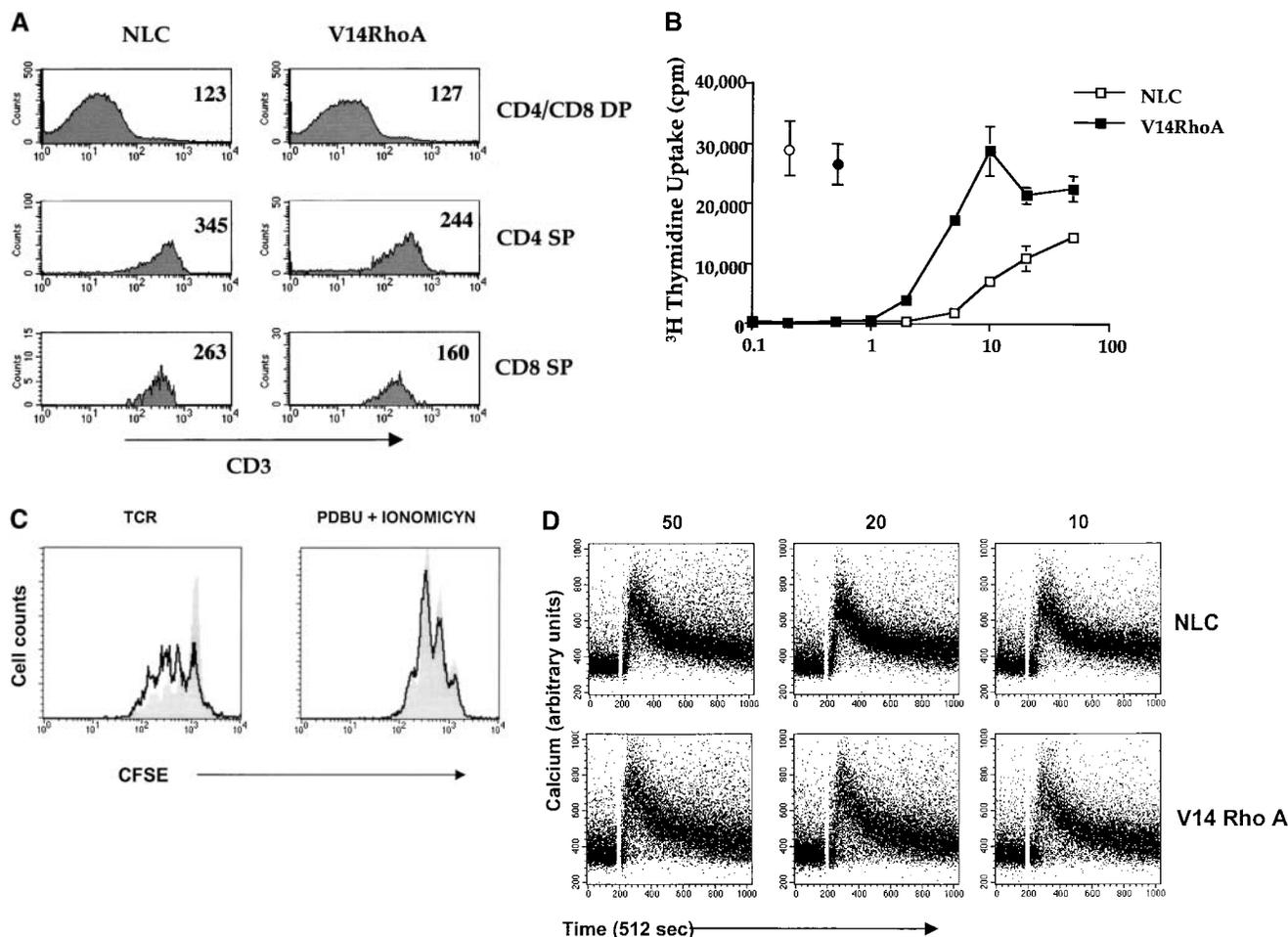
Figure 4. Mature thymocytes expressing constitutively active RhoA are hyperresponsive to TCR/CD3 triggering. (A) Analysis of CD3/TCR expression on thymic populations. Thymocytes from NLC and V14RhoA mice were stained for CD4, CD8, and CD3ε. Histograms show expression of CD3 on CD4$^+$CD8$^+$ DP population, CD4$^+$ single positive (CD4 SP), and CD8$^+$ single positive (CD8 SP). Mean fluorescence intensity for CD3 expression is shown in the histograms. (B) TCR-stimulated in vitro proliferation of thymocytes. Total thymocytes from NLC and V14RhoA transgenic mice were prepared as described previously. Proliferation of thymic T cells in response to a range of concentrations of plate-bound anti-CD3 antibody, or a combination of PdBu (5 ng/ml) and ionomycin (0.5 ng/ml), was assessed by the incorporation of [³H]thymidine during the last 12 h of a 36-h assay. Graph shows mean of [³H]thymidine incorporation (± SEM) of triplicate samples. Circles represent proliferation of thymocytes to PdBu plus ionomycin. The experiment shown is representative of three different experiments. White circles, NLC; black circles, V14RhoA. (C) CFSE analysis of cell division in TCR-stimulated CD4 SP thymocytes. CFSE-labeled thymocytes from NLC and V14RhoA transgenic mice were stimulated with soluble anti-CD3 antibodies or PdBu (5 ng/ml) and ionomycin (0.5 ng/ml) as described previously. After 48 h in culture cells were stained for CD4 and CD8 and analyzed by flow cytometry for CD4 and CD8 expression and CFSE fluorescence. Histograms show CFSE fluorescence gated on CD4 SP cells of NLC (shaded) or V14RhoA (open) mice. (D) TCR-stimulated intracellular calcium flux in CD4 SP thymocytes. Thymocytes were preloaded with Indo-1 coated with anti-CD3 antibodies and stained for CD4 and CD8. Intracellular calcium flux upon CD3 triggering was analyzed by flow cytometry as described in Materials and Methods. Cells were stimulated with goat anti–hamster, to crosslink the anti-CD3, at the concentrations and times indicated. Panels show intracellular calcium concentration in CD4 SP thymocytes as a ratio of Indo-1 violet/blue fluorescence versus time. Each channel on the time axis represents 500 msec.

point distal to the immediate biochemistry triggered by antigen receptors.

Activation of RhoA Potentiates TCR-α/β–induced Proliferative Responses In Vivo. In vitro proliferation experiments suggested that activation of RhoA could potentiate responses mediated by the mature TCR-α/β. Moreover, we had also noted that RhoA activation was associated with increased integrin-mediated cell adhesion and it has been proposed that cosignaling by integrins would facilitate TCR-mediated responses within the thymus (28). To examine the effect of RhoA signaling

on TCR-α/β–mediated responses in the thymus, we examined the effect of V14RhoA on positive and negative selection of thymocytes expressing TCR transgenes. The HY T cell receptor is specific for the male HY antigen presented by MHC class I H-2 D^b molecules (29). Thymocytes expressing the HY-specific TCR are negatively selected in male 2D^b mice and undergo massive deletion resulting in very few detectable DP and SP with a strong reduction in overall thymic cellularity. The expression of active RhoA did not change this phenotype (data not shown).

Thymocytes from female HY-TCR transgenic mice are positively selected on a H-2 D^b background and develop into mature CD8 SP cells. Strikingly, the thymic phenotype of V14RhoA/HY-TCR female double transgenic mice was markedly different from the normal phenotype seen in HY-TCR transgenic females. The data in Fig. 5 A (top) show that, in thymocytes from female mice that coexpress the HY-TCR and V14RhoA, there are markedly increased numbers of positively selected HY-TCR transgenic T cells. This was also seen in analysis of splenic populations (Fig. 5 A, bottom). To examine further the effects of RhoA on thymocyte selection, V14RhoA transgenic mice were bred with a different TCR transgenic line, F5-TCR transgenic mice. The F5 transgenic TCR recognizes

an influenza nuclear protein peptide in the context of H-2D^b (30) and thymocytes are positively selected to CD8 SP cells in mice with an H-2D^b background. The results in Fig. 5 B show that, in thymocytes and spleens coexpressing the F5-TCR transgene and V14 Rho, there are increased numbers of F5-TCR transgenic T cells. The analyses of the V14RhoA/HY-TCR and V14RhoA/F5-TCR double transgenic mice reveals that positive selection is more efficient in thymocytes expressing active RhoA.

Crosstalk between the GTPases Rac-1 and RhoA in Thymocyte Development. The inability of active RhoA to rescue pre–T cell differentiation in Rag-1^{−/−} mice is in marked contrast to the actions of a closely related GTPase Rac-1 (11). Rac-1 is activated by the guanine nucleotide
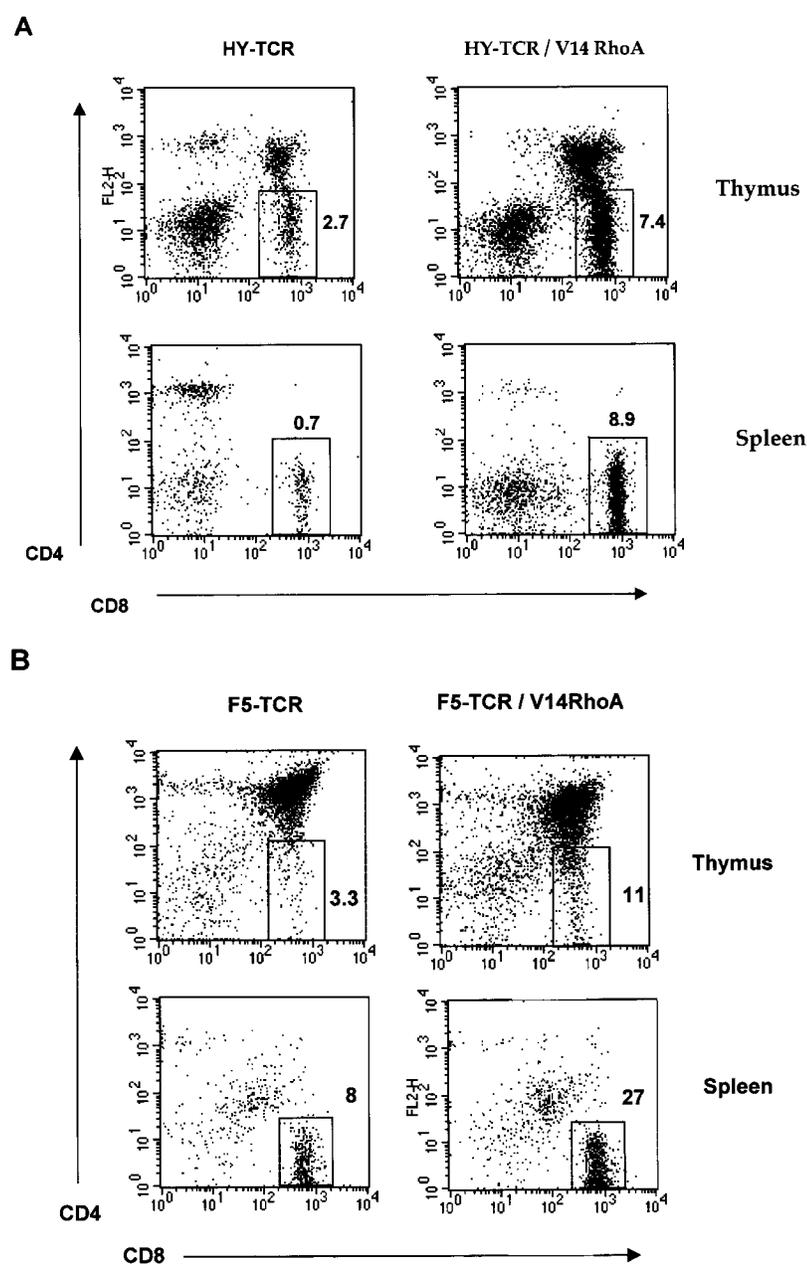

Figure 5. Activated RhoA enhances positive selection. (A) Analysis of thymic and splenic CD4/CD8 populations in female HY-TCR/V14RhoA double transgenic mice. Thymocytes and splenocytes of HY-TCR and HY-TCR/V14RhoA female mice were stained for CD4, CD8, and HY-TCR and analyzed by flow cytometry. Panels show CD4/CD8 profile gated on HY-TCR^high thymocytes (top) and splenocytes (bottom). Absolute numbers (10^6 cells) of CD8 SP thymocytes and splenocytes expressing high levels of HY-TCR are indicated. (B) Analysis of thymic and splenic CD4/CD8 populations in F5-TCR/V14RhoA double transgenic mice. Thymocytes and splenocytes of F5-TCR and F5-TCR/V14RhoA mice were stained for CD4, CD8, and Vβ11 and analyzed by flow cytometry. Panels show CD4/CD8 profile gated on Vβ11^high thymocytes and splenocytes. Absolute numbers (10^6 cells) of CD8 SP thymocytes and splenocytes expressing high levels of Vβ11 are indicated. Each two dimensional dot plot shown is representative of at least three independent experiments.

exchange protein Vav-1 (5) and mice lacking this protein have shown thymic hypocellularity associated with a pre-TCR block and inefficient negative and positive selection (9, 10). The developmental defects caused by loss of Vav-1 in pre-T cells can be corrected by expression of a constitutively active mutant of Rac-1 that regulates the actin cytoskeleton (11). In this context, V14RhoA and active Rac-1 have some common effects on actin dynamics (20, 31) and Vav-1 and Rac-1 are both able to induce RhoA–mediated changes in the actin cytoskeleton in in vitro models (31, 32). To explore physiologically relevant links between Rac-1 and RhoA in the thymus we examined the Rho dependency of Rac-1–induced pre-T cell differentiation and compared the ability of RhoA and Rac-1 to rescue T cell development in Vav-1 null mice.

To examine the Rho dependency of Rac-1–induced pre-T cell differentiation, CD2L61 Rac-1 transgenic mice expressing constitutively active Rac-1 were bred to mice in which the CD2-LCR was used to give pre-T cell expression of *Clostridium botulinum* C3 transferase, a selective inhibitor of Rho (14). In CD2-C3 transgenic mice, pre-T cell receptor expression occurs normally but further development is blocked at the DN3 cell stage. Thymi from CD2-C3 mice look like Rag$^{-/-}$ thymi in that they show a DN block at the DN3 stage and they fail to generate CD4CD8 DPs (14). Activation of Rac-1 can induce thymocyes to differentiate from CD4CD8 DNs to CD4CD8 DPs in Rag$^{-/-}$ thymocytes that lack a pre-TCR complex. The data in Fig. 6 A show that Rac-1 cannot induce CD4CD8 DPs in the absence of Rho function. Rac-1 also potentiates DN3/DN4 transition (11) as revealed by CD44/25 staining profiles of Rac-1 DNs; all cells in the DN3 gate have lower levels of CD25 than normal and are shifted into a population intermediate between the DN3 and DN4 stage. The ability of active Rac-1 to modulate DN3/DN4 transition is lost in CD2-C3 transgenic mice

(Fig. 6 B). Thus the actions of Rac-1 in pre-T cells are dependent on functional Rho signaling pathways.

Pre-T cell developmental defects in Vav-1 null mice can be rescued by expression of an activated mutant of Rac-1, L61Rac-1. Therefore, we determined if expression of V14RhoA could reverse the pre-T cell developmental defects caused by loss of Vav-1. Analysis of V14RhoA/Vav-1$^{-/-}$ mice showed that expression of the active RhoA mutant did not reconstitute cellularity in Vav-1$^{-/-}$ thymi (Fig. 7 A). Moreover, CD44/CD25 analysis revealed that V14RhoA/Vav-1$^{-/-}$ mice continue to be blocked at the DN3 stage of pre-T cell development and show a skewed accumulation of DNs and depleted DPs (Fig. 7 B and C). Defective antigen receptor signal transduction in Vav-1$^{-/-}$ thymocytes is reflected in its inability to upregulate CD5 expression in DPs, a marker of antigen receptor function. The loss of CD5 upregulation is restored by expression of active Rac-1 (11). The present data show that expression of V14Rho does not restore CD5 expression to normal in Vav-1$^{-/-}$ cells (Fig. 7 D). Vav-1$^{-/-}$ DPs ineffectively make SP mature T lymphocytes because of defects in positive and negative selection (10). Expression of active RhoA in Vav-1$^{-/-}$ did not regenerate SP thymocytes.

Discussion

This study uses a T cell–specific promoter to express an active Rho mutant, V14RhoA, in T cells of transgenic mice. This allowed direct analysis of the consequences of Rho activation for thymocyte development. Previously, it has been shown that Rho function is necessary for the development of pre-T cells; specifically for survival and proliferative responses controlled by the pre-TCR and the tyrosine kinase p56lck (12, 14, 19). RhoA was thus positioned downstream of the pre-TCR complex as part of the signals this receptor used to determine pre-T cell fate. This
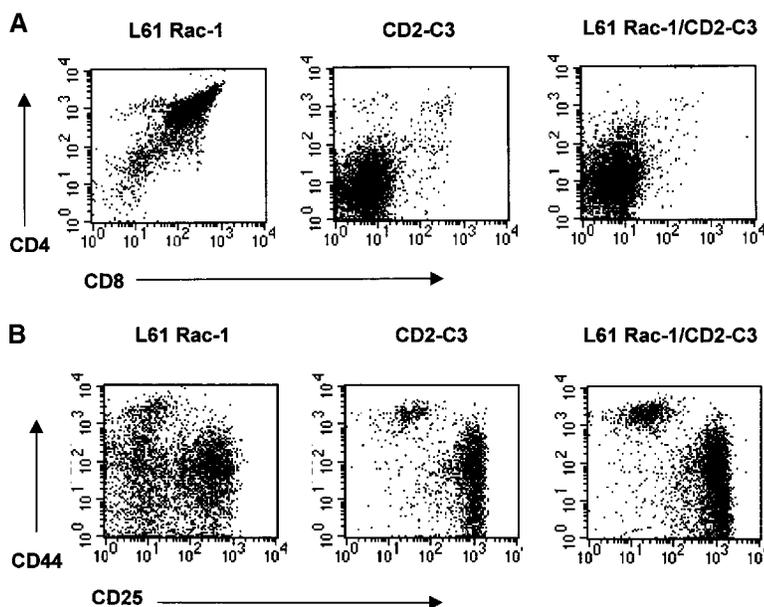

Figure 6. Differentiation of L61 Rac-1 pre-T cells is dependent of RhoA function. (A) Thymocytes from L61 Rac-1, CD2-C3, and L61 Rac-1/CD2-C3 transgenic mice were stained for CD4 and CD8, and analyzed by flow cytometry. Dot plots show CD4/CD8 profiles. Results are representative of three independent experiments. (B) Thymocytes from L61 Rac-1, CD2-C3, and L61 Rac-1/CD2-C3 transgenic mice were stained for CD25 and CD44, as described in Fig. 3 A, and analyzed by flow cytometry. Dot plots show CD44/CD25 profiles in lineage-null, Thy-1.2$^+$ cells. Results are representative of three independent experiments.
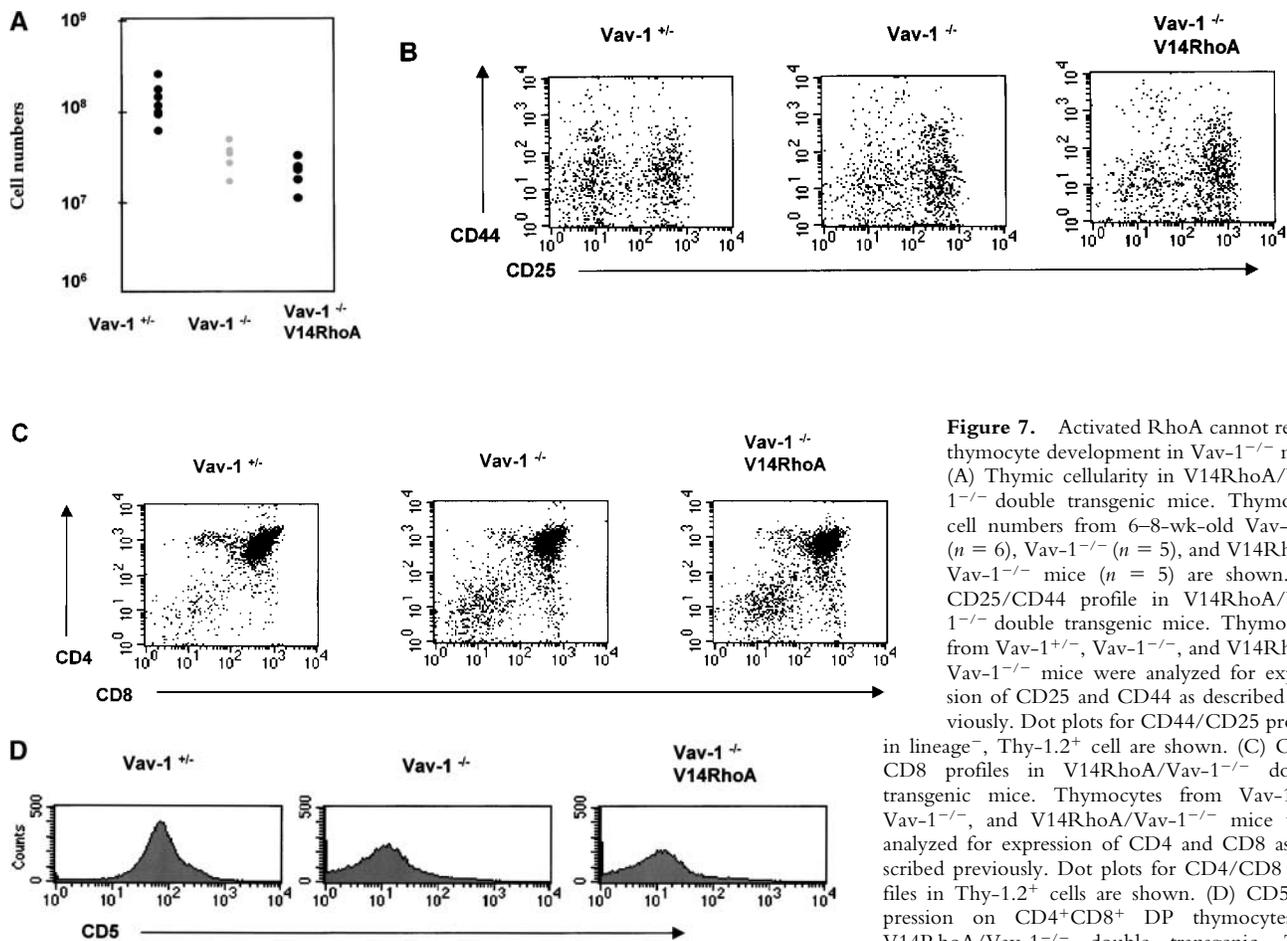
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Figure 7. Activated RhoA cannot rescue thymocyte development in Vav-1$^{-/-}$ mice. (A) Thymic cellularity in V14RhoA/Vav-1$^{-/-}$ double transgenic mice. Thymocyte cell numbers from 6–8-wk-old Vav-1$^{+/-}$ ($n = 6$), Vav-1$^{-/-}$ ($n = 5$), and V14RhoA/Vav-1$^{-/-}$ mice ($n = 5$) are shown. (B) CD25/CD44 profile in V14RhoA/Vav-1$^{-/-}$ double transgenic mice. Thymocytes from Vav-1$^{+/-}$, Vav-1$^{-/-}$, and V14RhoA/Vav-1$^{-/-}$ mice were analyzed for expression of CD25 and CD44 as described previously. Dot plots for CD44/CD25 profiles in lineage$^-$, Thy-1.2$^+$ cell are shown. (C) CD4/CD8 profiles in V14RhoA/Vav-1$^{-/-}$ double transgenic mice. Thymocytes from Vav-1$^{+/-}$, Vav-1$^{-/-}$, and V14RhoA/Vav-1$^{-/-}$ mice were analyzed for expression of CD4 and CD8 as described previously. Dot plots for CD4/CD8 profiles in Thy-1.2$^+$ cells are shown. (D) CD5 expression on CD4$^+$CD8$^+$ DP thymocytes in V14RhoA/Vav-1$^{-/-}$ double transgenic. Thymocytes from Vav-1$^{+/-}$, Vav-1$^{-/-}$, and V14RhoA/Vav-1$^{-/-}$ mice were analyzed for expression of CD4 and CD8 as described previously. CD5 expression was analyzed on CD4$^+$CD8$^+$ Thy1.2$^+$ cells. Results are representative of at least three independent experiments.

study now shows that activation of RhoA is able to induce integrin-mediated cell adhesion in thymocytes but is not sufficient to allow pre-T cell survival, differentiation, or proliferation in the absence of normal pre-TCR function. The fact that RhoA is required but not sufficient for pre-T cell differentiation means that RhoA must act in conjunction with other signaling molecules to determine the fate of early T cell progenitors.

Previous work on Rho function in T cell biology has inevitably focused on pre-T cells because the effects of losing Rho function in the thymi are so profound that very few DP or SP T cells develop and those that do, transform to form T cell lymphomas (33). The expression of V14RhoA was not associated with any increased tendency of mice to develop any malignancy (unpublished data). Moreover, the failure of V14RhoA to modulate pre-T cell differentiation means that it was possible to obtain DP and SP T cells expressing active RhoA in normal numbers and examine the impact of RhoA signaling for the first time in more mature T cell populations. One striking observation from these studies of RhoA action was that this GTPase can potentiate TCR-α/β responses. The potency of RhoA as an amplifier

of TCR responses can be seen in vitro, the SP T cells that differentiate in V14RhoA transgenic mice are hyperresponsive to TCR responses in terms of proliferation despite reduced TCR expression. Moreover, analysis of V14RhoA mice in the context of expression of two defined TCR transgenes revealed that RhoA activation strikingly augmented positive selection consistent with enhanced TCR responses in vivo.

The ability of RhoA to amplify responses mediated by the TCR-α/β complex was not seen in the context of pre-TCR responses; these were unperturbed by RhoA activation. The pre-TCR comprises the TCR-β subunit paired with the pre-Tα and the invariant CD3 chains (2). The pre-TCR does not have a ligand, rather its assembly at the plasma membrane in membrane microdomains is sufficient to initiate signal transduction for pre-T cell development (34–36). In DPs, the replacement of the pre-Tα subunit with the TCR-α chain generates the mature TCR-α/β which initiates signaling for selection when triggered by antigen/MHC complexes present on the surface of thymic epithelial cells. Cell–cell contacts mediated by integrins have been suggested to provide a selective boost to signal-

ing for positive selection of SPs in the thymus (28). This study shows that RhoA activation is able to promote cell adhesion in primary thymocyte populations. This would facilitate and stabilize contacts between the T cell and cells presenting antigen/MHC complexes and hence promote SP differentiation. The pre-TCR would not be sensitive to this RhoA-mediated phenomenon because it signals autonomously without the need for an exogenous ligand and hence without dependency on cell contact.

Previous studies have shown that the GTPase Rac-1 can drive significant pre-TCR differentiation in the absence of the pre-TCR in Rag-1$^{-/-}$ mice and augment pre-TCR responses in wild-type mice (11). Herein we show that Rho signaling is not sufficient to allow pre-T cell survival, differentiation, or proliferation in the absence of normal pre-TCR function, nor could V14RhoA mimic active Rac-1 and amplify pre-TCR signals. Nevertheless, Rac-1 actions in pre-T cells require Rho function. The actions of Rac-1 in pre-T cell differentiation thus involve RhoA but must require additional effectors. We have also noted that developmental defects caused by loss of the GEF Vav-1 in pre-T cells can be corrected by expression of activated Rac-1 that modulates actin dynamics (11). The effects of Rac-1 on actin structures are best studied in fibroblasts where it is noted that Rac-1 activation induces lammelipodia formation and then goes on to trigger RhoA–mediated actin metabolism that culminates in the formation of focal adhesion complexes (31, 32). In this respect, in transformed cell lines Rho and Rac have been reported to have a similar impact on the lymphocyte cytoskeleton (20). However, this report shows that only activation of Rac-1 can compensate for loss of Vav-1 in pre-T cells whereas activation of RhoA cannot. Rac-1 and RhoA thus do not have interchangeable functions in the thymus and there must be unique actions of Rac-1 on the cytoskeleton relevant to pre-T cell biology that cannot be triggered by RhoA.

In summary, this study extends our understanding of the importance of RhoA in the thymus and reveals that, besides its involvement in early thymocyte development, this GTPase also regulates cell adhesion in thymocytes and regulates thymocyte positive selection. This study also reveals Rho signaling is necessary but not sufficient to allow pre-T cell survival, differentiation, or proliferation in the absence of normal pre-TCR function. Finally, this study reveals signaling crosstalk between the GTPases Rac-1 and RhoA in the thymus. Rac-1–induced pre-T cell differentiation required Rho function but is not mediated solely by RhoA. It remains to be determined if Rac-1 and RhoA operate in linear or parallel pathways to control thymocyte development.

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