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Integrin Regulation by RhoA in Thymocytes

Susina Vielkind, Maighread Gallagher-Gambarelli,1 Manuel Gomez,3 Heather J. Hinton,4 and Doreen A. Cantrell5

The guanine nucleotide-binding protein Rho has essential functions in T cell development and is important for the survival and proliferation of T cell progenitors in the thymus. To explore the mechanisms used by RhoA to control thymocyte biology, the role of this GTPase in the regulation of integrin-mediated cell adhesion was examined. The data show that RhoA activation is sufficient to stimulate β1 and β2 integrin-mediated adhesion in murine thymocytes. RhoA is also needed for integrin activation in vivo as loss of Rho function impaired the ability of thymocytes to adhere to the extracellular matrix protein VCAM-1 and prevented integrin activation induced by the GTPases Rac-1 and Rap1A in vitro. The regulated activity of integrins is needed for cell motility and in the present study it was seen that RhoA activity is critical for integrin-mediated thymocyte migration to chemokines in vitro. Thus, RhoA has a critical role in regulating cell adhesion and migration during T cell development. The Journal of Immunology, 2005, 175: 350–357.

Thymocyte development is controlled by AgRs, chemokines, and cytokines. One key signaling molecule in the thymus is the guanine nucleotide-binding protein Rho. The importance of this GTPase for T cell development has been revealed by studies of transgenic mice that express Clostridium botulinum C3-transferase under the control of T cell-specific promoters in thymocytes (1–5). C3-transferase selectively ADP-ribosylates Rho within its effector-binding domain and thereby abolishes its biological function (6–9). Transgenic mice that express C3-transferase under the control of the p56
cx promoter have a thymus in which T cell progenitors and subsequent thymocyte subsets are devoid of Rho function. Studies of these mice show that Rho inactivation severely impairs the generation of T cells, resulting in a very small thymus and severely reduced numbers of peripheral T cells (1, 2, 5). The importance of RhoA in T cell development stems from its ability to mediate proliferative and differentiation signals induced by Src kinase p56
cx and the GTPase Rac-1 in pre-T cells (3, 10). Rho is also a critical component of survival signaling pathways in T cell progenitors (1, 2, 5). A complementary strategy to probe Rho function in the thymus has been to examine the effects of a gain of function mutant of RhoA in the thymus. Studies of transgenic mice with T cell-specific expression of an active RhoA mutant, V14RhoA, indicate that activation of RhoA potentiates AgR responses both in vivo and in vitro (10).

The molecular basis for the role of RhoA in the thymus is not known. However, recent studies revealed that RhoA activation in primary thymocytes is associated with increased β1 integrin-mediated cell adhesion (10). Integrins play an important role in T cell biology as their function is required for cell-cell adhesion and cell-extracellular matrix (ECM) contacts and will also participate in regulating migration of T cells (11–14). The predominant integrins on T cells are the β1 integrins VLA-4 (αvβ1), VLA-5 (αvβ5), and the β2 integrin LFA-1 (αLβ2) (15–18). These have low activity on resting lymphocytes but can be stimulated to mediate adhesion in response to a number of signals including AgRs and chemokines. These stimuli induce a process termed “inside-out” signaling which is typically associated with increases in integrin avidity for ligand as a result of increases in integrin lateral mobility and clustering and may also increase integrin affinity (19, 20). Once activated, integrins have a key role in coordinating cell motility and migration. The directed migration of thymocytes is essential for normal T cell development (21–23) and any action of RhoA on integrin function could influence the ability of thymocytes to make cell contacts and migrate and could explain why Rho is necessary for thymocyte differentiation. In this respect, LckC3 thymi have a very disorganized structure with no clear cortex or medulla which is compatible with a model in which Rho function is needed for normal thymocyte integrin activation and cell migration (1).

However, the role of RhoA in the control of cell motility is not ubiquitous but is cell state dependent with clear evidence for both RhoA-dependent and -independent mechanisms of cell motility in different cell populations (24). For example, it has been described in leukocytes that Rho function regulates chemokine-induced integrin activation and migration on ECM (25–27), whereas in other cells such as neutrophils, Rho is needed to promote cell detachment from the ECM (28). Similarly, there is considerable plasticity in leukocytes.

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6 Abbreviations used in this paper: ECM, extracellular matrix; BCECF-AM, 2’,7’- bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; PdBu, phorbol 12, 13-dibutyrate; NLC, normal littermate control; SDF-1, stromal cell-derived factor-1c; Teck, thymus-expressed chemokine; FN, fibronectin; DN, double negative; DP, double positive; SP, single positive; HSA, heat-stable Ag; ROCK Rho-associated kinase.
in the role of RhoA in integrin regulation. Initial studies using leukemic T cell lines concluded that RhoA stimulation was not the signal for integrin activation and implicated Rac-1, another Rho family GTPase, and the GTPase Rap1 to be important for this process (29–31). Moreover, it was reported that in human T lymphoblasts, inhibition of RhoA potentiates integrin-mediated cell adhesion (28, 32). Conversely, in pre-B cells and lymph node-derived lymphocytes, RhoA was reported as necessary for chemokine-induced integrin activation and cell motility (25, 26). One reason for these contradictory results may be that the role of Rho as a regulator of integrins and cell motility is not universal but is restricted to certain cell subpopulations. Different routes to activate integrins may thus vary in their requirement for RhoA.

The aim of the present study was to explore the ability of RhoA to regulate integrin-mediated responses and cell motility in primary mouse thymocytes. As well, the role of Rho in integrin activation induced by the GTPases Rac-1 and Rap1A was examined. The data show that activation of RhoA is sufficient to induce integrin-mediated adhesion in mouse thymocytes and peripheral T cells. Moreover, Rho function was shown to be necessary for in vivo activation of integrins by Rac-1 and Rap1A. Finally, we provide evidence that Rho signaling is important for chemokine-induced thymocyte migration.

Materials and Methods

Transgenic mice

Mice were bred and maintained under specific pathogen-free conditions at the Cancer Research U.K. Biological Resources Unit. Transgenic mice expressing the constitutively active mutant of RhoA, V14RhoA; the constitutively active Rac-1 mutant, L61Rac1; and the constitutively active Rap1A mutant, V12Rap1A, in the thymus and peripheral T cell compartments under the control of the human CD2 promoter and locus control region were generated as previously described (10, 33–35). Lck3 C3 transgenic mice which selectively express the bacterial toxin C3-transferrase in the thymus have been described in detail elsewhere (1–3, 5, 36).

Cell preparation and culture

Murine thymi and spleens were isolated freshly by dissection from 4- to 8-week-old animals. Tissue was disaggregated by passing through a fine mesh filter to obtain a single-cell suspension. Cell numbers were determined by trypan blue exclusion of dead cells and were adjusted according to the subsequent application by centrifugation (5 min; 1400 rpm) and resuspension in medium. For growing up T lymphoblasts, splenocyte cell numbers were adjusted to 5 × 10⁶ cells/ml in RPMI 1640/0.1% FCS/5 μM 2-ME and the cells were stimulated with soluble anti-CD3 mAb (145-2C11) at 10 μg/ml. After 36 h 2C11 was washed off and the T lymphoblasts were grown in rIL-2 (20 ng/ml; Chiron).

Cell attachment assays

Flat-bottom Maxisorp 96-well plates (Nunc) were either left uncoated (controls) or were coated overnight at 4°C with the indicated concentrations of the specific ligand in PBS; human plasma fibronectin (Sigma-Aldrich); the 120-kDa α-chymotryptic fragment of human fibronectin (Chemicon International); murine ICAM-1-Fc or VCAM-1-Fc (R&D Systems) and nonspecific binding sites were blocked with 2% denatured BSA (PBS) for 1 h at 37°C. Subsequently the wells were washed once with PBS and once with DMEM/25 mM HEPES (Invitrogen Life Technologies). Freshly isolated thymocytes were adjusted to 5 × 10⁶ cells/ml in assay medium (RPMI 1640/0.5% BSA) and 100 μl (1 × 10⁶ cells) were added to the top of the transwell (input cells) after removing the blocking solution. A total of 600 μl of the chemokine (recombinant human stromal cell-derived factor-1α (SDF-1α)/CXCL12 at 20 nM, or recombinant mouse thymus-expressed chemokine (Teck)/CCL25 at 3 μg/ml; R&D Systems) in assay medium was added to the bottom well. Cells were left to migrate for 4 h at 37°C. The cells that migrated to the bottom well were analyzed further. FACS calibrate beads (BD Biosciences) were used for quantifying the migrated cells using CellQuest software. The ratio of cells per bead was determined: number of events of cells vs number of events of beads in a two-dimensional dot plot. By referring to the total amount of beads that had been added to each sample, the total amount of cells was determined for each sample. Thymocyte subpopulations were analyzed using CD4, CD8α, and Thy1.2 Abs. Dead cells were identified using 7-aminominoacridine D staining (7 μg/ml).

Transwell migration assays

Transwell membranes (5 μm; Corning Costar) were coated overnight at 4°C with 2 μg/ml fibronectin (100 μg in PBS). Subsequently, the ligand-coated solution was removed by pipetting and 100 μl of the blocking solution (2% BSA/PBS) was added to the transwell membrane (1 h, 37°C). Freshly isolated thymocytes were adjusted to 1 × 10⁵ cells/ml in assay medium (RPMI 1640/0.5% BSA) and 100 μl (1 × 10⁶ cells) were added to the top of the transwell (input cells) after removing the blocking solution. A total of 600 μl of the chemokine (recombinant human stromal cell-derived factor-1α (SDF-1α)/CXCL12 at 20 nM, or recombinant mouse thymus-expressed chemokine (Teck)/CCL25 at 3 μg/ml; R&D Systems) in assay medium was added to the bottom well. Cells were left to migrate for 4 h at 37°C. The cells that migrated to the bottom well were analyzed further. FACS calibrate beads (BD Biosciences) were used for quantifying the migrated cells using CellQuest software. The ratio of cells per bead was determined: number of events of cells vs number of events of beads in a two-dimensional dot plot. By referring to the total amount of beads that had been added to each sample, the total amount of cells was determined for each sample. Thymocyte subpopulations were analyzed using CD4, CD8α, and Thy1.2 Abs. Dead cells were identified using 7-aminominoacridine D staining (7 μg/ml).

Flow cytometric analysis

Abs (BD Pharmingen) were conjugated either to FITC, PE, allophycocyanin, or biotin. Biotinylated Abs were revealed using streptavidin-TriColor (Caltag Laboratories). Thymocytes were stained for the surface expression of the following markers using the Abs given in parentheses: CD4 (RM-4), CD8α (53-67), Thy1.2/C90.2 (53-2.1), CD24/HS A (M1/69), CD262L/le-Selectin (MEL-14). Cells were stained with saturating concentrations of Ab at 4°C for 20 min at 1 × 10⁶ cells per sample in FACS buffer (DMEM (–) Phenol Red/25 mM HEPES/1% FCS). Cells were washed in this buffer between incubations and analysis was conducted on a FACSCalibur (BD Biosciences). Data were analyzed using CellQuest software (BD Biosciences).

Results

RhoA activation is sufficient for β1 and β2 integrin-mediated thymocyte adhesion

Experiments conducted on cultured lymphocyte cell lines in vitro have led to the conclusion that activation of RhoA is not sufficient for integrin activation (29, 37). Thymocytes express the β1 integrins VLA-4 and VLA-5 and the β2 integrin LFA-1 (15–18). Recently, we have shown that expression of a gain-of-function mutant of RhoA (V14RhoA) in transgenic thymocytes induces their adhesion to fibronectin (FN) (10). Whether this reflects equal activation of the β1 integrins VLA-4 and VLA-5 in T lymphocytes has not yet been explored. It was also not yet investigated whether RhoA required for integrin activation in vivo in primary thymocytes. Accordingly, we examined the adhesion of normal thymocytes and thymocytes expressing V14RhoA to selective β1 and
$\beta_2$ integrin ligands. In these experiments, T cells were isolated from transgenic mice with T cell-specific expression of V14RhoA, a constitutively active mutant of RhoA, or normal littermate control animals. Multiple cell binding sites have been characterized within the FN molecule and the 120-kDa $\alpha$-chymotryptic fragment of FN only comprises the Arg-Gly-Asp-Ser binding site that is recognized specifically by VLA-5 (38). Therefore, to address VLA-5 adhesion, the 120-kDa $\alpha$-chymotryptic fragment of FN was used as an integrin ligand. To examine VLA-4 activity, thymocyte adhesion to VCAM-1, the VLA-4 ligand which is expressed by stromal cells of the thymic cortex (39), was studied.

The data in Fig. 1A show that normal thymocytes have low levels of adhesion to the VLA-5-specific ligand, the 120-kDa chymotryptic fragment of FN, but this adhesion is clearly enhanced by expression of V14RhoA. Normal thymocytes have relatively high basal levels of adhesion to the VLA-4 ligand VCAM-1 (18) compared with their adhesion to FN (Fig. 1B) but at submaximal ligand concentrations this adhesion is also enhanced by expression of V14RhoA (Fig. 1C). The data show that the enhanced cell adhesion of V14RhoA thymocytes is not maximal but can be further stimulated when cells are activated with phorbol ester (Fig. 1C). The effects of RhoA on integrin activity in thymocytes are not restricted to $\beta_1$ integrins. LFA-1 is a cell adhesion molecule that belongs to the $\beta_2$ integrin subfamily ($\alpha_\beta_2$) that binds to ICAM-1 (19). The data in Fig. 1D show that thymocytes expressing active RhoA have higher levels of basal adhesion to the LFA-1 ($\alpha_\beta_2$) ligand ICAM-1. Again, this activation was not maximal as stimulation of cells with PdBu could further augment ICAM-1 adhesion in V14RhoA cells (Fig. 1E). The data in Fig. 1, F–H, show that surface levels of $\beta_2$ and $\beta_2$ integrins were similar on normal and V14RhoA thymocytes. V14RhoA in primary thymocytes induces increased activity of three classes of integrins on thymocytes, $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_\beta_2$.

Activation of RhoA stimulates integrin-mediated adhesion in peripheral T cells and a variety of thymocyte subsets

The experiments in Fig. 1 looked at the impact of RhoA activation on the adhesion responses of total thymocytes. However, the thymus is a mixture of T cells at different stages of development and maturation. Accordingly, it was decided to look at the impact of RhoA on cell adhesion of different thymocyte subsets to explore whether the integrin-stimulating functions of RhoA are restricted to certain cell populations. Thymocytes can be classified into individual subpopulations which are distinguished phenotypically on the basis of CD4 and CD8 coreceptor expression: immature CD4$^{-}$CD8$^{-}$ double-negative (DN) cells make the transition to mature CD4$^{+}$ or CD8$^{+}$ single-positive (SP) cells via an intermediate CD4$^{+}$CD8$^{-}$ double-positive (DP) stage. The results in Fig. 2A show that activation of RhoA stimulates adhesion to FN in CD4$^{+}$CD8$^{-}$ DP thymocytes. The data further show that RhoA could activate cell adhesion in SP thymocytes although this effect was not as strong as the action of RhoA in DPs. Normal DN thymocytes have high levels of basal adhesion to FN, which could not be effectively augmented by expressing active RhoA. Phorbol ester treatment resulted in an increase in the attachment of all normal and V14RhoA transgenic thymocyte subsets compared with basal adhesion levels. Interestingly, the high basal adhesion of DNs to FN could be stimulated further when cells were treated with PdBu indicating that integrin activity on DNs, although high, is not maximal; it is less susceptible to further stimulation by activation of RhoA but can be activated by phorbol ester.

Previous studies that failed to see integrin stimulation in response to RhoA activation were conducted in transformed lymphoid cell lines growing exponentially in tissue culture (29, 37). It is possible to activate primary T cells polyclonally and let them expand in vitro as T lymphoblasts for short time periods. Therefore, we were able to probe whether V14RhoA was able to induce integrin activation in these nontransformed in vitro-maintained T lymphoblasts. Splenocytes from normal or V14RhoA transgenic mice were activated polyclonally with anti-CD3 mAb and expanded for 5 days in IL-2. The data in Fig. 2B compare cell adhesion of normal and V14RhoA T lymphoblasts to FN. The basal adhesion to the $\beta_3$ integrin ligand FN is low in normal T lymphoblasts but high in blasts expressing V14RhoA. V14RhoA induced adhesion is not maximal, it can be further stimulated by treatment with phorbol ester (Fig. 2C). In summary, the data show that in the thymus expression of constitutively active RhoA stimulates adhesion in SP and particularly DP subsets and moreover, we found

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Activation of RhoA (V14RhoA) stimulates $\beta_1$ and $\beta_2$ integrin-mediated adhesion of thymocytes. Data show cell adhesion of thymocytes from V14RhoA transgenic mice and NLC. A, Integrin VLA-5-mediated adhesion to increasing concentrations of the 120-kDa $\alpha$-chymotryptic fragment of human FN. B, Adhesion of normal thymocytes was assayed to increasing concentrations of either FN or VCAM-1. C, Cell adhesion to 2 $\mu$g/ml VCAM-1 assayed in the presence and absence of 50 ng/ml PdBu. D, Cell adhesion to increasing concentrations of ICAM-1. E, Cell adhesion to ICAM-1 was assayed in the presence and absence of PdBu (50 ng/ml). F–H, Overlay FACS histograms of normal and V14RhoA thymocytes stained for LFA-1 (CD11a), VLA-4 (CD49d), and VLA-5 (CD49e), respectively. Experiment A is representative of three independent assays. All other results were obtained in two independent experiments that have been conducted in triplicate.
that activation of RhoA also enhances adhesion in peripheral T lymphocytes.

Rho regulates thymocyte adhesion to VCAM-1

It has been suggested that migrating lymphoid progenitors use a cellular matrix consisting of a subset of cytokeratin+ cortical stromal cells that express the integrin ligand VCAM-1 for intrathymic precursor adhesion and migration (39). CD4+CD8+ DP thymocytes normally have relatively high basal levels of cell adhesion to VCAM-1 (18), reflecting activation of VLA-4 in situ in the thymus (Fig. 1B). To judge whether losing Rho function prevented in vivo responses to integrin activation, we looked at the impact of losing Rho function on VCAM-1 adhesion. The key to these experiments was the availability of LckC3 transgenic mice where the Rho inactivator, C3-transferase, is expressed under the control of the p56Lck T cell-specific CD2 promoter to create mice that are double-transgenic for C3-transferase and active Rac-1 (L61Rac-1/LckC3). These results show that the ability of thymocytes to optimally activate VLA-4 in situ requires Rho function.

Rho function is necessary for in vivo activation of integrins by the GTPase Rac-1

To further probe the role of Rho in integrin activation in vivo, we examined the requirement for Rho in integrin activation induced by the GTPase Rac-1. There is a large body of evidence indicating that the GTPase Rac-1 is a key inducer of integrin activation in lymphocytes (29, 34, 40, 41). In cultured lymphocytes, in vitro expression of active Rac-1 can stimulate integrin-mediated cell adhesion (29). More importantly, there is strong evidence that Rac-1 stimulates integrin activity in thymocytes as thymocytes lacking expression of Vav-1, the guanine nucleotide exchange factor for Rac-1, have defective integrin activation (40, 41). In addition, expression of L61Rac-1, a constitutively active Rac-1 mutant in transgenic thymocytes, is sufficient to potentiate integrin activity in these cells (34). The functions of Vav-1 and Rac-1 in the thymus have been ascribed to the actions of these molecules on integrin function and, significantly, it has been shown that Rac-1 regulates thymocyte development partly via Rho-dependent pathways (10). However, it has not been addressed whether activation of integrins via Rac-1 is Rho dependent. To examine potential cross-talk between Rac-1 and Rho for integrin activation in vivo, LckC3 transgenic mice were bred onto transgenic mice that express a constitutively active mutant of Rac-1, L61Rac-1, under the control of the T cell-specific CD2 promoter to create mice that are double-transgenic for C3-transferase and active Rac-1 (L61Rac-1/LckC3). LckC3 thymus are very small (10% of normal thymocyte numbers) due to the impact of losing Rho function on pre-T cell survival and proliferation (1, 2). This phenotype was not changed in the double-transgenic L61Rac-1/LckC3 mice (data not shown) confirming that expression of active Rac-1 cannot induce thymocyte proliferation in the absence of Rho function (10). The data in Fig. 4A show the impact of Rac-1 activation on integrin-mediated cell adhesion to the β1 integrin ligand FN in thymocytes. As demonstrated previously, thymocytes expressing L61Rac-1 have high basal integrin activity compared with normal littermate control cells (Fig. 4A) (34). To analyze the impact of loss of Rho function on Rac-1-mediated adhesion, adhesion of L61Rac-1 thymocytes and thymocytes doubly transgenic for C3-transferase and active Rac-1 to FN was compared. Fig. 4B summarizes the data from three separate experiments and shows that L61Rac-1/LckC3 thymocytes have a greatly reduced ability to adhere to FN compared with cells expressing active Rac-1. L61Rac-1/LckC3 thymocyte adhesion levels to FN are in the range of wild-type cells; hence, the ability of active Rac-1 to stimulate integrin-mediated cell adhesion requires Rho function.
Rho function is necessary for optimal in vivo activation of integrins by the GTPase Rap1A

Another GTPase Rap1 has also emerged recently as an important regulator of integrins in a variety of cell lineages including primary T cells (30, 35, 42, 43). Rap1 accumulates in its active GTP-bound state in response to AgR or chemokine receptor triggering in lymphocytes and this biochemical process is necessary for integrin-mediated cell adhesion in activated T cells (44–46). Moreover, experiments using gain-of-function mutants of Rap1 have shown that this GTPase is sufficient for integrin activation in T and B cells (31, 43–49). The role of Rap1A in the thymus has also been studied using transgenic mice with T cell-specific expression of V12Rap1A, a constitutively active mutant of Rap1 (35). Experiments with these mice have established that Rap1A is a potent activator of both β1 and β2 integrin-mediated tyrosine adhesion in vivo. To investigate potential crosstalk between Rho and Rap1A in integrin regulation in an in vivo model, the V12Rap1A mice were bred onto the LckC3 transgenic mice that have lost Rho function in their T cells. The resultant mice, doubly transgenic for C3-transferase and active Rap1A (V12Rap1A/LckC3), were analyzed for their integrin function.

V12Rap1A thymi are normal in terms of size and cell content (35) whereas LckC3 thymi are very small (10-fold reduction in thymus cellularity) (1, 2). V12Rap1A/LckC3 thymi are phenotypically indistinguishable from LckC3 thymi (data not shown). Hence, activation of Rap1A cannot compensate for loss of Rho function and restore thymocyte proliferation or thymus size in LckC3 thymi. Ex vivo thymocytes that express a constitutively active mutant of Rap1, V12Rap1A, have very high basal levels of integrin activation compared with normal thymocytes (Fig. 5A). However, thymocytes that express active Rap1A on a Rho inactive background (V12Rap1A/LckC3) show a clear reduction in their ability to adhere to FN when compared with V12Rap1A-expressing cells (Fig. 5B). In experiments with active Rac-1 (Fig. 4B) loss of Rho function reduced integrin-mediated cell adhesion to wild-type levels, whereas it was a consistent result that loss of Rho function did not completely ablate the V12Rap1A effects. Rho function is thus needed for optimal integrin-mediated cell adhesion induced by active Rap1A, but there is a residual Rap1 response that appears to be insensitive to Rho inhibition.

The role of Rho in regulating thymocyte chemotaxis

Integrins are important for cell motility, as the process of cell migration requires that cells constantly form new attachments and release old attachments to move on an ECM. Our results show that RhoA regulates integrin activity in primary thymocytes, particularly in Rac-1 and Rap1A pathways, and prompted us to address whether RhoA is important for thymocyte chemotaxis and cell migration. This is a pertinent question, as any defects in cell migration in the thymus might explain why loss of Rho function is so deleterious for thymocyte development. There is evidence that Rho is important for the chemotaxis of mature leukocytes but recent studies have shown that closely related cell populations can vary in their dependence on Rho pathways for the regulation of cell migration (24, 50). To address whether thymocyte migration is Rho dependent, thymocytes from LckC3 transgenic mice were tested for their ability to undergo directed movement toward SDF-1α or Teck, chemokines known to regulate the trafficking of thymocytes (22, 51–53). The results in Fig. 6 show that inhibition of Rho results in a clear impairment of thymocyte migration. The inhibitory effect of loss of Rho function was observed for both SDF-1α and Teck.
Evidence that Rho inhibition blocks thymocyte emigration in vivo

The defective migratory capacity of LckC3 transgenic thymocytes in vitro raises the issue of whether there is a key role for Rho in regulating thymocyte migration in vivo. One marker of defective thymocyte migration is an abnormal frequency of mature SP thymocytes in the thymus which occurs as a result of blocked thymocyte emigration (23, 54). Indeed, although LckC3 thymocytes are greatly reduced in numbers due to the importance of Rho for pre-T cell survival and proliferation (1, 2), in terms of percentage, there is a relative enrichment of SPs with high levels of TCR expression in the thymus (Fig. 7A). Heat-stable Ag (HSA) is a well-characterized marker for assessing the maturation status of T cells (55, 56). HSA levels are very high on DN thymocytes, DP thymocytes begin to down-regulate HSA, and down-regulation is continued in CD4+ and CD8+ SP thymocytes. In mature peripheral T cells, HSA levels are lowest. The results in Fig. 7B show that CD4+CD8+ DP thymocytes from LckC3 transgenic mice express similar levels of HSA as DP thymocytes from normal mice. In contrast, mature CD4+ SP and CD8+ SP thymocytes in LckC3 transgenic mice showed a striking difference in HSA expression levels compared with normal mice. Specifically, SP thymocytes from mice that have lost Rho function (LckC3) have markedly down-regulated HSA levels, similar to those found expressed on mature peripheral T cells rather than recently selected SP thymocytes. Another marker to assess the maturation status of thymocytes is L-selectin (CD62L). L-selectin levels are low on immature thymocytes and are up-regulated as cells mature. Further analysis of the SP populations in the thymus revealed an evident increase in the number of mature L-selectinhigh cells in transgenic mice that have lost Rho function (Fig. 7C).

Discussion

The present study has examined the role of RhoA in regulating integrin activation in mouse T cells using in vivo models. Experiments with an active RhoA mutant V14RhoA indicate that RhoA activation is sufficient for integrin activation in thymocytes and peripheral T cells. The actions of active RhoA mimic the effects of activating the GTPases Rac-1 and Rap1A in thymocytes – effective activation of β1 and β2 integrins. Moreover, the present study shows that Rho activity is required for integrin activation induced by Rac-1 and Rap1A. The present data thus reveal a cross-talk between Rac-1, Rap1A and Rho for integrin activation, with Rho at a convergence point of the signaling pathways that regulate integrins in thymocytes.

The role of Rho in integrin regulation described in the present study may reflect unique aspects of integrin regulation in thymocytes. Hence, in vitro studies using transformed leukocyte cell lines concluded that RhoA was not necessary for integrin activation and only implicated Rac-1 or Rap1 in this process (29, 31, 37, 48). Furthermore, in some leukocytes, inhibition of RhoA can increase cell adhesion by preventing lymphocyte detachment (32). In thymocytes there are no discernable increases in basal integrin-mediated cell adhesion associated with loss of Rho function; rather, loss of Rho function reduces the ability of thymocytes to adhere to integrin ligands. These discrepancies must reflect that different cell types use different mechanisms for integrin regulation. Indeed, the present data show that different T cell subsets vary in their response to active RhoA in terms of their ability to activate integrins. RhoA efficiently activated integrin-mediated cell adhesion in CD4+CD8+ DP thymocytes and peripheral T cells but not in CD4+CD8− DN thymocytes. Hence, different lymphocyte subsets may differ in their repertoire of Rho effectors or in the downstream “wiring” of effectors.

Integrin-mediated cell adhesion is important for cell migration and evidence is presented herein that Rho function is necessary for chemokine-induced migration in thymocytes. Previous studies have shown a requirement for Rho function for the migration of lymphocytes (57–60) but the role of Rho in thymocyte migration has not previously been addressed. In this context it should be emphasized that not all directed cell migration requires signaling through Rho. For example, studies of migrating tumor cells in a three-dimensional matrix suggested that there are Rho-dependent and Rho-independent modes of cell motility (24). A “rounded mode” of cell migration was associated with signaling through RhoA and its downstream effector Rho-associated kinase (ROCK) in contrast to an “elongated mode” of cell movement that is driven by Rac and is independent of Rho activity. Tumor cells can thus switch between different signaling pathways and mechanisms to

FIGURE 7. Effect of loss of Rho function on in vivo migration of mature thymocytes. Data shown are flow cytometric analysis of thymocytes from NLC and LckC3 transgenic mice. A, Two-dimensional dot plots show flow cytometric analysis of total thymocytes. Cells were stained with anti-CD4 and anti-CD8. The percentage of each thymocyte subset is shown in each dot plot quadrant. Data are representative of more than three experiments. B, Overlay histograms show HSA expression on thymocyte subpopulations. Cells were stained for CD4, CD8 and HSA. Similar results were obtained in three independent experiments. C, Overlay histograms show CD62L expression on thymocyte subpopulations. Cells were stained for CD4, CD8, and CD62L.
regulate cell migration, and it is therefore possible that different lymphocyte populations also use different mechanisms.

T cell development in the thymus is regulated by interactions between stromal cells and lymphoid progenitors and integrins are important for mediating these cell/cell contacts and regulating cell migration (61, 62). Indeed, recent studies have shown that normal thymocytes have high basal levels of VLA-4-mediated adhesion (18) and it has been proposed that the chemokine receptor CXCR4 and its ligand SDF-1α/CXCL12 induce directed migration of T cell progenitors over a matrix of VCAM-1-expressing stroma (22, 39). CXCR4 has been shown to be necessary for correct localization of T cell progenitors in the thymus and in the absence of this chemokine receptor, T cell progenitors fail to differentiate (22, 53). CXCR4 signaling is required for proper localization of T cell precursors upon entry into the thymus and probably is also needed for directed outward migration of T cell precursors to the cortex, a process during which they receive the environmental signals required for survival. Our study shows that the ability of thymocytes to adhere to a VCAM-1 matrix is reduced in cells lacking Rho function and is enhanced in cells expressing active Rho. Furthermore, analysis of the chemotactic response of thymocytes to the chemokines SDF-1α or Teck revealed that Rho function is necessary for thymocytes to move directionally along a chemotactic gradient. In the in vitro data that Rho is needed for thymocyte migration are also supported by in vivo observations. Hence, thymi from LckC3 transgenic mice have a very disorganized structure with no clear cortex or medulla, consistent with the model that Rho function is necessary to allow thymocytes to migrate to their normal intrathymic location (1). It is well-documented that thymocytes lacking Rho function have defective survival responses in vivo (1, 2). A failure of thymocytes lacking Rho function to migrate correctly could be responsible for these survival defects, as mislocalized thymocytes may not make contact with the appropriate thymic stroma, which could result in their failure to receive appropriate survival signals. The importance of RhoA for SP migration might also explain the relatively high percentage of SP T cells in the thymus of LckC3 mice compared with normal thymus. These data are thus consistent with a model whereby the few SPs that develop in LckC3 mice are retained in the thymus. Previous studies have defined two points at which Rho can influence cell motility. In one model, RhoA and its effector ROCK are required for the disruption of cell attachments to the ECM at the rear of the cell (28, 59, 60). In the second model, RhoA is necessary for the inside-out activation of integrins regulated by chemokines in primary leukocytes (25, 26). There are a number of different cell types where the first model is applicable and where Rho/ROCK signaling regulates cell detachment, notably in monocytic cell lines, Jurkat T leukemic cells, and human effector T cells (28, 59, 60). The characteristic of these cells is that the inhibition of Rho function results in the failure of cells to detach from ECM resulting in increases in cell adhesion. In contrast, we observed that thymocytes lacking Rho function show decreased, not increased, cell adhesion. Hence, the migration deficiencies of LckC3 thymocytes could be explained by a failure of these cells to attach to ECM in the first place and may not be caused by a failure of the cells to detach. The present results showing a Rho requirement for Rap1A and Rac-1 regulation of integrins in thymocytes are thus more consistent with the second model, namely, that RhoA is part of the inside-out signaling mechanisms that control integrin function and ECM attachments in response to extracellular stimuli.

In summary, the present study shows that RhoA activation is sufficient for integrin activation in thymocytes and is necessary for integrin activation induced by the GTPases Rap1A and Rac-1. Integrins are crucial molecules for cell migration as this is a multi-step process that is dependent on the ability of a cell to attach to a particular surface, and subsequently, to detach from it to move forward. The reduced ability of thymocytes lacking Rho function to activate integrins is thus reflected by the inability of these cells to migrate efficiently in vitro and in vivo. The present data thus establish RhoA as a key signaling molecule that controls integrin function and cell motility, explaining why this GTPase is so important for thymocyte development. The RhoA effector(s) that are involved in regulating integrin-mediated migration in thymocytes and the pathway(s) that mediate Rho-dependent integrin responses of the GTPases Rac-1 and Rap1 are issues for future investigation.

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Disclosures

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References


