Rho GTPase Cdc42 is essential for human T-cell development

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ABSTRACT

Background
Rho GTPases are involved in the regulation of many cell functions, including some related to the actin cytoskeleton. Different Rho GTPases have been shown to be important for T-cell development in mice. However, their role in human T-cell development has not yet been explored.

Design and Methods
We examined the expression and activation of Rho GTPases along different stages of T-cell development in the human thymus. Early stage human thymocytes were transduced with constitutively active and dominant negative mutants of different Rho GTPases to explore their role in human T-cell development, as analyzed in fetal thymus organ cultures. The use of these mutants as well as Rho GTPase-specific inhibitors allowed us to explore the role of GTPases in thymocyte migration.

Results
We found that the expression of several Rho GTPases is differently regulated during successive stages of T-cell development in man, suggesting a specific role in human thymopoiesis. In chimeric fetal thymus organ culture, T-cell development was not or only mildly affected by expression of dominant negative Rac1 and Rac2, but was severely impaired in the presence of dominant negative Cdc42, associated with enhanced apoptosis and reduced proliferation. Kinetic analysis revealed that Cdc42 is necessary in human T-cell development both before and after expression of the pre-T-cell receptor. Using inhibitors and retrovirally transferred mutants of the aforementioned Rho GTPases, we showed that only Rac1 is necessary for migration of different thymocyte subsets, including the early $CD34^+$ fraction, towards stromal cell-derived factor-1α. Constitutively active mutants of Rac1, Rac2 and Cdc42 all impaired migration towards stromal cell-derived factor-1α and T-cell development to different degrees.

Conclusions
This is the first report on Rho GTPases in human T-cell development, showing the essential role of Cdc42. Our data suggest that enhanced apoptotic death and reduced proliferation rather than disturbed migration explains the decreased thymopoiesis induced by dominant negative Cdc42.

Key words: lymphopoiesis, Rho GTP-binding proteins, Rac GTP-binding proteins, hematopoietic stem cells, T lymphocytes.

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Introduction

The family of Rho GTPases comprises a major subgroup of the Ras superfamily of small GTPases. RhoA, Rac1 and Cdc42 are the best characterized members of this family of at least 23 genes. Most Rho GTPases cycle between an active GTP-bound and an inactive GDP-bound state. In the GTP-bound conformation, Rho GTPases are able to interact with downstream effectors, thereby initiating diverse signaling cascades. Because each Rho GTPase can recognize multiple effectors, and some effectors are recognized by more than one Rho GTPase, these interactions generate a complex network.[1-4]

Cycling of Rho GTPases is tightly regulated by different types of regulatory molecules, including Rho guanosine nucleotide exchange factors, Rho GTPase activating proteins and Rho GDP dissociation inhibitors.

Rho GTPases are involved in the regulation of many cell functions, sometimes cell type-specific, including a number of functions related to the actin cytoskeleton: gene transcription, cell cycle progression, survival, adhesion, migration, cell polarity, enzymatic activities, and axon guidance.[1,3,4] These functions can be both overlapping and unique to different Rho GTPases, which complicates interpretation of loss-of-function studies.

Rac1, Cdc42 and RhoA are ubiquitously expressed, whereas Rac2 is restricted to the hematopoietic system.[5] In the past 10 years, efforts have been made to elucidate the specific roles of Rho GTPases in murine T-cell development using gain- and loss-of-function strategies. The size and cellularity of thymi lacking functional Rho are drastically reduced.[6] Thymocyte-specific expression of C. *botulinum* C3 exoenzyme, an inhibitor of RhoA, B and C, results in a survival defect of early thymocyte progenitors and $CD4^+CD8^+$ double-positive cells.[7,8] Thymocyte development is not perturbed in $Rac2^{-/-}$ mice and the effect on T-cell development is limited in conditional $Rac1^{-/-}$ mice.[9-11] However, the generation of conditional $Rac1^{-/-}Rac2^{-/-}$ double knock-out mice showed that proliferation, apoptosis, adhesion and migration of thymocytes were disturbed, revealing a crucial but redundant role of Rac1 and Rac2.[10] This was confirmed by a recent study that used an alternative approach to create conditional double knock-out mice, which, in addition, linked the results to altered Notch signaling.[12] A dominant negative mutant of Rac1 was used to demonstrate that Rac1 is required for the generation of CD4 single-positive cells from a murine double-positive cell line by preventing apoptosis.[13] Loss of function of Cdc42 causes *in utero* death. Conditional knock-out mice for this Rho GTPase were recently generated and used to study hematopoietic stem cells but, so far, the effect on T-cell development has not been studied.[14,15]

Expression of constitutively active RhoA promotes positive selection and generates hyper-responsive mature T cells.[16] Transgenic mice expressing constitutively active Rac1 in the thymus have revealed a role for Rac1 as a positive regulator of β selection.[17] However, development into single-positive cells is not possible because of exacerbated negative selection.[18] Thymocyte-specific expression of constitutively active Rac2 and Cdc42 results in severely reduced thymic cellularity because of the deletion of double-positive cells, which could be explained by the induction of apoptosis.[19,20] Although the importance of Rho GTPases in T-cell development is well established, the mode of action is not clear. In the case of Rho, it was suggested that the inability of thymocytes lacking Rho function to migrate correctly could explain why Rho is necessary for T-cell development.[21] It has been shown that directed migration is essential for T-cell development. During their journey through the thymus, developing thymocytes encounter specific microenvironments that provide the appropriate signals for a particular stage of T-cell development, such as cell surface molecules, secreted proteins and extracellular matrix components.[22] It is, therefore, plausible that factors disturbing migration may also disturb development as cells fail to make the necessary cell-cell contacts. Besides migration, (pre-) T-cell receptor (TCR) signaling, which affects thymic selection by balancing survival against apoptosis and which induces proliferation, might explain the essential role of Rho GTPases.

In this study we investigated the importance of different Rho GTPases in human T-cell differentiation.

Design and Methods

Monoclonal antibodies and reagents

The mouse anti-human monoclonal antibodies used were CD4-allophycocyanin (APC) or phycoerythrin (PE) (SK3), CD34-APC (8G12), HLA-DR-APC (L243), CD3-PE or fluorescein isothiocyanate (FITC) (SK7), Ki-67-PE (B56) and CD8-FITC (SK1), all from Becton Dickinson Immunocytometry Systems (BDIS, Erembodegem, Belgium); CD8β-PE (2ST8.5H7) from Coulter (Miami, FL, USA); CD1-biotin (OKT 6), unlabeled CD3 (OKT 3) and CD8 (OKT 8) from American Type Culture Collection (ATCC, Rockville, MD, USA); anti-glycophorin-A was a kind gift from Dr. L. Lanier (University of California, San Francisco, CA, USA). Stem cell factor (SCF) and interleukin (IL)-7 were from R&D Systems (Abingdon, UK), recombinant human stromal cell-derived factor-1 (SDF-1α/CXCL12) was from Peprotech (London, UK). The Rho GTPase inhibitors used were Rac inhibitor NSC23766 (Calbiochem, Nottingham, UK) and secramine A.[23]

Cell purification

Child thymus tissue, removed during cardiac surgery, was obtained and used following the guidelines of the Medical Ethical Commission of Ghent University Hospital. Informed consent was provided according to the Declaration of Helsinki. Immature single-positive (ISP4) cells were purified from total thymocytes by immunomagnetic depletion using antibodies against glycophorin-A, CD3 and CD8 and sheep anti-mouse Dynabeads (Dynal Biotech, Hamburg, Germany) according to the manufacturer's instructions. The enriched population was labeled with CD4-PE, CD34-APC, CD8-, CD3- and HLA-DR-FITC and the $CD34^-CD4^+FITC^-$ fraction was sorted on a FACS Vantage (BDIS) using CellQuest software (BDIS). Double-positive cells were sorted from total thymocytes after labeling with CD3-FITC, CD4-APC and CD8β-PE. For isolation of mature $CD3^+$, single-positive cells and $CD34^+$ cells, thymus mononuclear cells were isolated over a Lymphoprep density gradient (Axis-Shield PoC AS, Oslo, Norway). For single-positive cells, this was followed by depletion of $CD1^+$ cells using CD1-biotin and Streptavidin MicroBeads (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were subsequently labeled with CD3-FITC, CD4-APC and CD8β-PE, and sorted for either $CD3^+CD8^+$ or $CD3^+CD4^+$ cell subsets.

Purity of all these thymocyte subsets was at least 98.4%. CD34$^+$ thymus cells were enriched by positive selection using either CD34 MACS (Miltenyi Biotec) or EasySep (StemCell Technologies SARL, Grenoble, France). The purity of the collected cells was on average 95.3±3.3%.

Constructs, viral production and transduction

All plasmid constructs were made into the retroviral vector LZRS-IRES-EGFP as described before.[24] Dominant negative mutants Rac1N17, Rac2N17 and Cdc42N17 (placenta isoform), and constitutively active mutants Rac1V12 and Rac2V12 were obtained from the UMR cDNA Resource Center (www.cdna.org) and *EcoRI-Xho*I transferred from pcDNA3.1+ to LZRS-IRES-EGFP. Cdc42V12 (placenta isoform) was *BamH*I transferred from pEBG-Cdc42V12/GST to LZRS-IRES-EGFP.[25] Direct sequencing (ABI, Foster City, CA, USA), western blotting or a pull-down based Rho GTPase activity assay (G-LISA™, Cytoskeleton, Denver, CO, USA) was used as instructed by the respective supplier and using standard protocols to confirm the integrity of all constructs and to measure RhoA, Rac1, total Rac (Rac1+Rac2+Rac3) and Cdc42 activity in thymocyte subsets.

For the production of retroviral supernatant, the Phoenix-Amphotropic packaging cell line was transfected with LZRS-IRES-EGFP (control) and LZRS-(insert)-IRES-EGFP plasmids using calcium-phosphate precipitation.[26] Viral supernatants contained between 3.0×10^6 and 15.5×10^6 transducing units per mL titrated on 293T cells (ATCC). For chemotaxis and fetal thymus organ culture, CD34$^+$ thymus cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL) and 10% heat-inactivated fetal calf serum (complete IMDM, Invitrogen) in the presence of SCF (5 ng/mL) and IL-7 (10 ng/mL) for 24 h prior to transduction. After transduction, performed as previously described,[24] cells were cultured with the same concentrations of cytokines for an additional period of 1 day prior to fetal thymus organ culture or 2 days prior to chemotaxis.

Real-time polymerase chain reaction

After sorting of thymocyte subsets, cells were resuspended in TRIzol (Invitrogen) and stored at -70 °C until use. Total RNA was extracted from thymocytes as instructed by the supplier of TRIzol (Invitrogen), and then DNase-treated (DNase I, Invitrogen) and reverse transcribed (Reverse Transcription Core Kit, Eurogentec, Seraing, Belgium). Genes assayed included RhoA (Sybr Green I detection, Eurogentec), Rac1, Rac2 and Cdc42 (TaqMan detection chemistry, Eurogentec). For normalisation, YWHAZ mRNA was chosen from ten housekeeping genes based on GENORM (Sybr Green I detection, Eurogentec).[27] Primers (Invitrogen) and Taqman probes (Eurogentec) were described previously[27-29] or were designed with Primer Express 2.0 software (Applied Biosystems, Foster City, CA) for RhoA (forward CGGAATGATGAGCACACAAGG; reverse ATGTACCCAAAAGCGCCAATC). Primer specificity was confirmed with plasmids expressing one of the Rho GTPases. Quantitative real-time PCR was performed on an ABI Prism 7300 Sequence Detection System (Applied Biosystems) as described before.[29] Comparative quantification of the target gene expression was performed based on the standard curve method. To compare different donors, donor subsets were normalized to own total mean relative expression.

Fetal thymus organ culture

Chimeric fetal thymus organ culture and subsequent flow cytometric analysis at day 21 were performed as described previously.[30,31] The total progeny of 10^4 pre-cultured CD34$^+$ or ISP4 cells, transduced 24 h earlier, was seeded into each lobe using the 'hanging drop' method. The excess of transduced progenitor cells, which were not used for the hanging drop, were kept in culture to determine transduction efficiency after 2 or 4 days. The mean transduction efficiency of different viruses varied between 8±3% and 26±4%. After 14 days, half of the medium of the fetal thymus organ culture was replaced. The mean number of human cells per thymic lobe harvested after 21 days of culture and starting from control transduced cells was 408×10^3 cells (range, 190×10^3 to 880×10^3 cells) (n=10). This large variation can be attributed to differences between human donors and murine thymic lobes. However, the fraction of transduced cells was previously shown to be very reproducible, which allows quantification of thymic development using the thymocyte generation ratio, i.e., the ratio of the fraction of EGFP$^+$ thymocytes harvested to the fraction of EGFP$^+$ progenitors that were put in the fetal thymus organ culture.[31] The thymocyte generation ratio does not take into account the total number of cells generated in each lobe, but compares the development of transduced cells to that of non-transduced cells, the latter serving as an internal control since they are derived from the same donor and cultured in the same thymic lobe.[31]

Chemotaxis

Chemotaxis assays were performed in duplicate using 5 μm pore filters (Transwell, 24 well cell cluster, Corning Costar, Cambridge, MA, USA). Migration medium [600 μL IMDM supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL) and 0.5% bovine serum albumin (Sigma-Aldrich, Bornem, Belgium)] containing 50 ng/mL SDF-1α was added to the lower compartment; 100 μL of cell suspension in migration medium without SDF-1α (between 1.2×10^6 and 5.0×10^6 cells/mL for total thymus single cell suspensions and between 2.0×10^5 and 1.7×10^6 cells/mL for sorted CD34$^+$ cells) were placed in the upper well. In experiments with inhibitors for Rho GTPases, total thymus or CD34$^+$ thymus cells were cultured overnight in complete IMDM with SCF (5 ng/mL) and IL-7 (10 ng/mL) and NSC23766 (200 μM for total thymocytes or 100 μM for CD34$^+$ cells) or secramine A (2 μM). These inhibitors were added at the same concentration to the upper well during migration. Transwells were incubated for 3 h at 37°C in 7% (v/v) CO_2. Upper wells were removed and cells migrated into the lower compartment were harvested after addition of a fixed amount of Flow-Count Fluorospheres (Beckman Coulter, Fullerton, CA, USA). In experiments starting from total thymocytes, migrated cells were stained with CD4-APC, CD8,-PE and CD3-FITC. Flow cytometry was done on a FACS®Calibur (BDIS) to determine the absolute number of input and migrated cells as well as the relative frequencies of specific subsets of thymocytes in initial and migrated populations. The fraction of migrating cells (e.g. double-positive thymocytes) was calculated as follows: (% double-positive cells in migrated population x total amount of migrated cells) / (% double-positive cells in initial population x total amount of input cells). For each subset, migration was evaluated relative to migration of the same population in the absence of an inhibitor. When thymus CD34$^+$ cells were transduced with mutants of Rho GTPases, mean transduction efficiency was 17%, giving us the opportunity to use the non-transduced EGFP$^-$ cells, present in each well, as an internal control for migration. In this case, relative migration was calculated as follows: fraction of migrating EGFP$^+$ cells / fraction of migrating EGFP$^-$ cells, where fraction of migrating cells is calculated as indicated above. The per-

centage of migration typically fluctuates around 25% for both total and CD34+ cells.

Statistical analysis

Fetal thymus organ culture and chemotaxis data were analyzed using the paired sample *t* test (SPSS, version 12; SPSS, Chicago, IL, USA). *P* values less than 0.05 were considered statistically significant.

Results

Expression of Rho GTPases in the thymus

Whereas Rac1, Cdc42 and RhoA are ubiquitously expressed in mammalian tissue, Rac2 expression is restricted to the hematopoietic system. We examined the expression pattern of Rho GTPases in different stages of thymocyte development to gain information on their potential role in human T-cell development. Rac1 and RhoA are broadly expressed, with highest levels in the early stages of T-cell development (CD34+, ISP4) (Figure 1). Rac2 expression is upregulated during development towards single-positive cells and Cdc42 is expressed in very early (CD34+) as well as in late (single-positive) stages of development. Because the activity of Rho GTPases is the result of post-transcriptional regulation by Rho guanosine nucleotide exchange factors, GTPase activating proteins and dissociation inhibitors, we used a pull-down-based Rho GTPase activity assay (G- LISA™, Cytoskeleton; *data not shown*) to examine activity in different thymocyte subsets. We found RhoA and Rac1 activity in most fractions (CD34+, ISP4, double-positive and CD3+) comparable to that of unseparated thymocytes (±50%). The Cdc42 G-LISA was not sensitive enough to reproducibly measure Cdc42 activity. Different expression and activation patterns reflect modulation during T-cell development, suggesting a role for Rho GTPases in human thymocyte development.

Human T-cell development is disturbed by deregulating the activity of Rho GTPases

To investigate the role of Rho GTPases in human T-cell development, we used an *in vitro* chimeric fetal thymus organ culture, the best validated *in vitro* model for human T-cell development at the moment.[32] Fetal thymus organ culture has previously been shown to support development of retrovirally transduced CD34+ and immature CD3-CD4+CD8- single-positive ISP4 cells.[30] A flowchart to explain the fetal thymus organ culture experiment is presented in Figure 2. The percentage of EGFP+ cells after the culture, corrected for initial transduction efficiency (thymocyte generation ratio), is a parameter for T-cell development. It allows comparison of the effect of the transgene to untransduced cells, and is not blurred by individual differences between donors or between murine thymic lobes. A large set of data has shown that the median thymocyte generation ratio of control transduced cells is well above one.[31] This indicates that transduced cells have a slight advantage over non-transduced cells in this assay. Given that retroviral vectors are used for the gene transfer, this suggests that cycling cells (after 24 h of culture in the presence of appropriate cytokines) within the CD34+ fraction are more potent T-cell precursors. As the lentiviral vectors expressing shRNA against Rho GTPase family members described before[29] expressed only transiently in fetal thymus organ culture, human CD34+ thymus cells were retrovirally transduced with dominant negative or constitutively active mutants of Rho GTPases, and assayed for T-cell development. Dominant negative Cdc42 severely impairs T-cell development, as measured by the thymocyte generation ratio (Figure 3A). The few T cells that were generated while expressing dominant negative Cdc42 displayed a normal phenotype (Figure 3C) and the relative frequencies of double-positive and single-positive cells were comparable to those of control transduced cells (Figure 3C and *data not shown*). The absence of Rac1 or Rac2 activity resulted in only very small albeit statistically significant effects on thymopoiesis.

All constitutively active mutants disturb T-cell development (Figure 3B). Although the relative number of transgene-expressing cells was reduced, the percentage of double-positive cells or CD3+ cells was comparable between EGFP+ and EGFP- cells (Figure 3D). In the CD3+ population, no differences in the fractions expressing TCRαβ or TCRγδ were observed between EGFP+ and EGFP- cells (*data not shown*). However, we observed a positive correlation between transgene expression level (measured by EGFP) and CD3 expression levels for cells transduced with constitutively active Rac1, Rac2 or Cdc42 but not with control (Figure 3D).

Cdc42 is essential from early stages of human T-cell development

Among all Rho GTPases tested, manipulation of Cdc42, whether this was mediated by expression of constitutively active or dominant negative mutants, resulted in the most profound effect on T-cell development. As Cdc42 has been shown to be important in different processes that could be relevant in the context of T-cell development, such as proliferation, apoptosis and migration, we examined whether

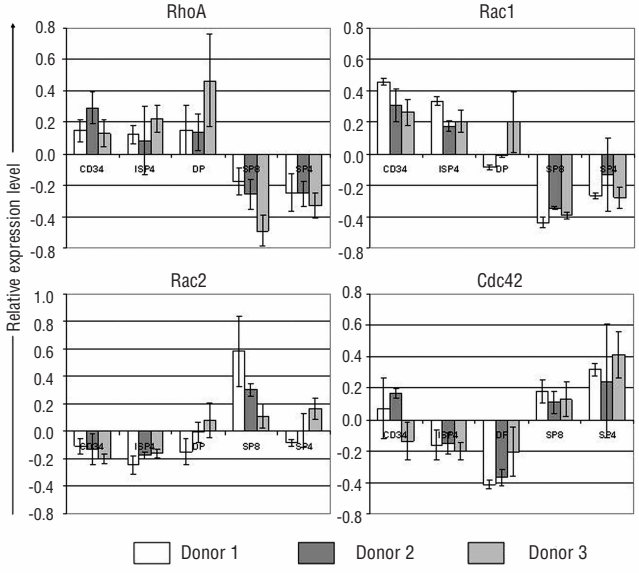

Figure 1. Expression pattern of Rho GTPases in human thymus. Real time PCR analysis of sorted thymocyte subsets. Bars indicate mRNA expression level of the indicated gene relative to the housekeeping gene YWHAZ for the corresponding subset. For comparison of the three donors, levels of individual subsets were normalized to mean donor expression overall subsets, designated zero. Error bars indicate standard deviation.

any of these processes could be linked to its mechanism of effect on T-cell development. Both thymus CD34⁺ and ISP4 cells transduced with control vector or mutant Cdc42 were cultured in medium supplemented with cytokines (SCF and IL-7) or used to initiate T-cell development in fetal thymus organ culture. Cell culture, initiated with transduced CD34⁺ cells, for more than 1 week resulted in comparable amounts of Cdc42N17-expressing cells and control transduced cells (% of EGFP⁺ cells after 8 days of culture / % of EGFP⁺ cells after 4 days of culture: 0.92±0.08 and 1.01±0.14 for Cdc42N17 and control-transduced cells, respectively; n=3). By contrast, T-cell generation from these progenitors was greatly reduced in fetal thymus organ culture already after 4 days (Figure 4). This was observed both with CD34⁺ progenitors and with ISP4 thymocytes, the latter known to be in part TCR-β selected.[33] Cells transduced with the constitutively active mutant of Cdc42 hampered T-cell development only after longer culture periods (Figure 4, beyond day 8), suggesting that the effect of Cdc42 overactivity on T-cell generation arises only later in development.

To explore whether apoptosis was induced and proliferation was hampered in the absence of functional Cdc42, we performed additional fetal thymus organ culture experiments.

Firstly, we analyzed the fraction of annexin V⁺ and 7-AAD⁺ cells in fetal thymus organ culture initiated with Cdc42N17 and control transduced cells. Double-positive annexin V⁺ 7-AAD⁺ dead and annexin V⁺ 7-AAD⁻ apoptotic cell fractions were increased in the presence of Cdc42N17 (Figure 4D).

Secondly, the proliferation marker Ki-67 was stained in these cultures. As shown in Figure 5, both at day 4 and day 21 of fetal thymus organ culture, Cdc42N17-transduced thymocytes showed a reduced fraction of Ki-67 positive cells (less than 10%) compared to control-transduced cells (up to 20%). The observation that transduced cell numbers are not reduced after cell culture of CD34⁺ thymocytes, but are drastically reduced in fetal thymus organ culture over the same period, indicates a thymus-specific effect of dominant negative Cdc42 on CD34⁺ cell proliferation. Cdc42 is necessary early in T-cell development as well as past the point of pre-TCR expression, as both CD34⁺ and ISP4 progenitor cells expressing dominant negative Cdc42 show reduced T-cell generation.

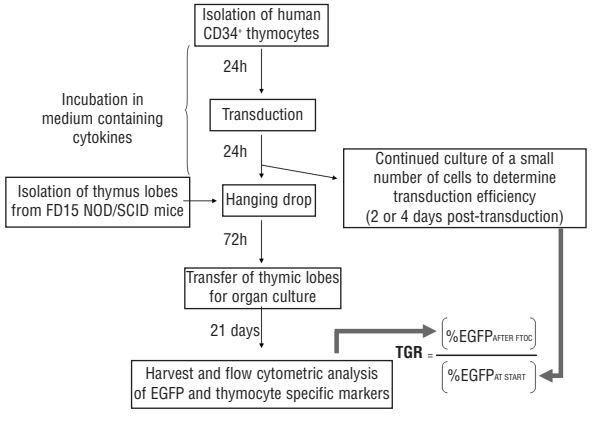

Figure 2. Flow-chart of fetal thymus organ culture. TGR: thymocyte generation ratio.

The effects of Rho GTPases on migration and T-cell generation are not correlated

Migration is crucial for T-cell development. Rho GTPases have been shown to be important for migration of many cell types, including hematopoietic stem cells[14,15,34-37] and thymocytes[21] in the mouse. We, therefore, examined the *in*

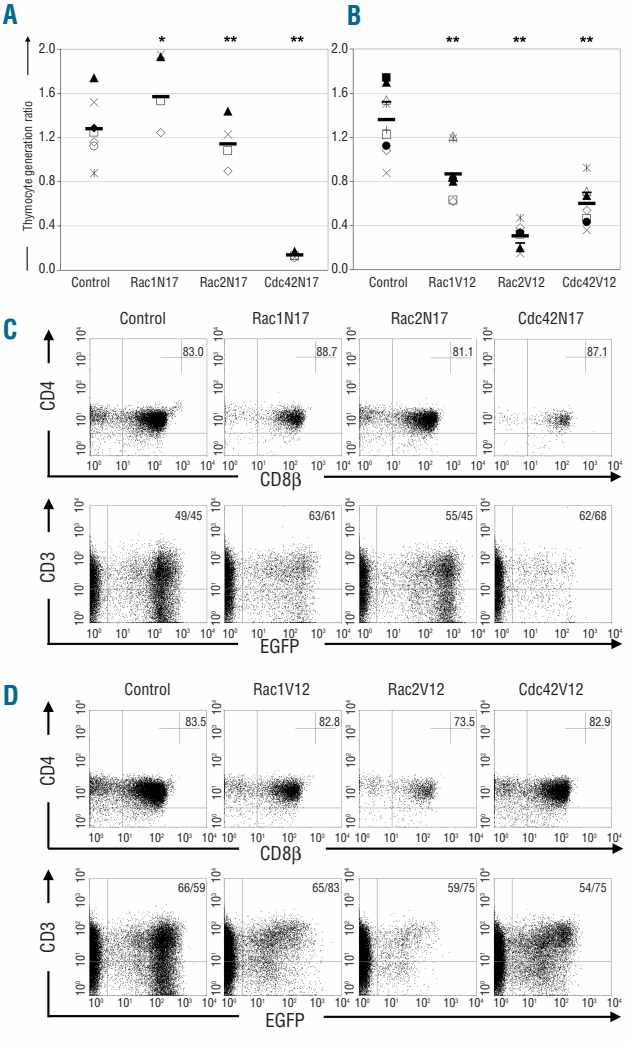

Figure 3. Influence of expression of mutant Rho GTPases on human T-cell development. (A-B) Overview of T-cell development as evaluated in fetal thymus organ culture. Cells transduced with dominant negative mutants (A) and constitutively active mutants (B) of Rho GTPases. Thymocyte generation ratio was calculated as indicated in the 'Design and Methods' section. Within each graph, each symbol represents data derived from the same thymus donor. The mean is represented by the thick horizontal line. Asterisks indicate statistically significant differences between mutant Rho GTPase and control (*P<0.05; **P<0.01). (C-D) Representative flow cytometric analysis of control, dominant negative mutant (C) and constitutively active mutant (D) Rho GTPase transduced cells analyzed after 21 days of fetal thymus organ culture. Upper panels are gated on EGFP⁺ fraction, lower panels (EGFP versus CD3) are gated on total human cells. Figures indicate the fraction of double-positive cells (upper dot plots) and mean fluorescence intensity of CD3 for EGFP⁻ (left) and EGFP⁺ (right) cells (lower dot plots). Quadrants are set according to isotype controls.

vitro migration capacity of different thymocyte subsets after manipulation of Rho GTPase activation by treatment with different inhibitors. NSC23766 is a rationally designed Rac specific inhibitor that interferes with binding to specific guanosine nucleotide exchange factors.[38] The recently described small molecule inhibitor secramine A blocks Cdc42 activation in a RhoGDI1 dependent manner.[23] In the absence of inhibitors, the migration response to SDF-1α differs between thymocyte subsets. The immature $CD4^-CD8^-$ cells are more responsive towards SDF-1α (43.5±19.1%) than are total thymocytes (29.5±15.7%, mean percentage ± standard deviation, n=7; *P*=0.062), which is in agreement with previously reported data on murine thymocytes.[39] We analyzed the migration of T-cell subsets in the presence of an inhibitor relative to the migration of these subsets in the absence of the inhibitor. The Rac specific inhibitor NSC23766 reduced migration to SDF-1α of total thymocytes and of each subset (Figure 6A). Secramine A does not interfere with chemotaxis. As $CD34^+$ progenitor cells migrate in response to SDF-1α and these precursors were used to initiate T-cell development in our experiments, we specifically analyzed migration of sorted child thymus $CD34^+$ cells using chemical inhibitors and gene transfer of constitutively active and dominant negative mutants of different Rho GTPases.[40] The influence of Rho GTPase inhibitors on the migration capacity of child thymus $CD34^+$ cells was comparable to that on the other thymocyte subsets (Figure 6A). All constitutively active mutants impaired migration of $CD34^+$ cells to SDF-1α but only Rac1 seemed to be essential as all other dominant negative mutants showed normal migration (Figure 6B-C). By gating on intermediate and strong $EGFP^+$ cells, reflecting intermediate and strong transgene expression, a dose response was evident for the mutants that affect migration. These results confirm the data derived from experiments with inhibitors and suggest that the inhibitory effect of NSC23766 on migration of $CD34^+$ cells is mediated by Rac1 but not Rac2. Collectively, these data suggest that Rac1, but not Rac2 or Cdc42, is essential for thymocyte migration towards SDF-1α. The essential role of Cdc42 for human T-cell development we show in this study is, therefore, unlikely to be mediated by a role in thymocyte migration.

Discussion

With this study, we show that Cdc42 is necessary for T-cell development. Cdc42 has been shown to be important for hematopoietic stem cell quiescence and retention in the bone marrow in conditional knock-out mice, but no data are available on the effect of loss-of function of Cdc42 in T-cell development.[15] However, Wiskott-Aldrich syndrome patients, who have mutations in the activated-Cdc42 binding protein WASP, show impaired T-cell development and function.[3-5] Progenitor cells transduced with dominant negative Cdc42 sustain in *in vitro* culture with cytokines, but not in fetal thymus organ culture for the same period, showing that Cdc42 is specifically necessary for T-cell development.

As inhibition of migration was considered a potential mechanism of action of mutant Rho GTPases, we investigated this possibility. However, migration towards SDF-1α was not impaired after treatment of thymocytes with the

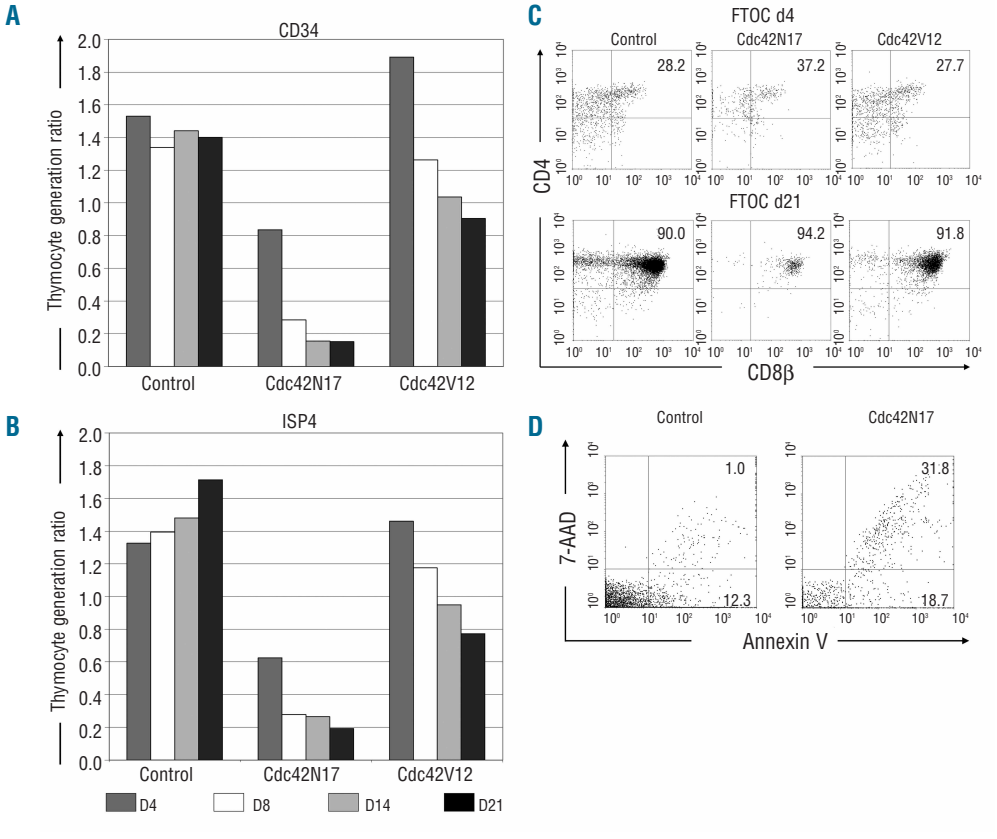

Figure 4. Kinetic analysis of T-cell development of progenitor cells expressing Cdc42 mutants. (A-B) Fetal thymus organ culture started from transduced $CD34^+$ cells (A) or ISP4 cells (B), analyzed at different time points. Bars represent thymocyte generation ratio and results shown are representative of at least two independent experiments. (C-D) Representative flow cytometric analysis after 4 days (C; upper panel) and 21 days (C; lower panel) or 14 days (D) of fetal thymus organ culture started from $CD34^+$ cells. Gates are set on human $EGFP^+$ cells. The figure indicates the percentage of cells in the corresponding quadrant. Quadrants are set according to isotype controls. Results shown are representative of at least two independent experiments.

Cdc42 inhibitor secramine A, or after transduction of the dominant negative mutant of Cdc42. We also ruled out the possibility that Cdc42N17-transduced cells are unable to enter the thymic lobe during hanging drop, as transduced cells were not enriched in the fraction not entering the lobe (*data not shown*). Thymocyte generation from the ISP4 population, expressing in part a pre-TCR,[33] was also greatly reduced due to dominant negative Cdc42 expression. Pre-TCR signaling cannot, therefore, overcome the effect of dominant negative Cdc42. Although we could not reliably measure Cdc42 activity in thymocyte CD34$^+$ fractions or in ISP4, these results show that the mutant protein competed in sufficient amounts for Cdc42-specific guanosine nucleotide exchange factors in ISP4 cells.

Another process that could be affected is proliferation. Our observation that T-cell development was hampered overall, with no obvious block at a specific developmental stage and unaltered subset ratios is in agreement with the hypothesis that proliferation is hampered when Cdc42 cannot be activated, possibly in relation to β-selection. Indeed, the ratio of double-positive to single-positive thymocytes generated was normal, but the cells were greatly reduced in number. Moreover, Ki-67 staining showed that thymocytes expressing dominant negative Cdc42 had a reduced proliferating fraction. The signal transduction pathways downstream of the pre-TCR which lead to induction of proliferation have not been fully elucidated yet, but a role for cyclin D3 was recently demonstrated in mice and humans.[41,42] Cdc42 is involved in G1 cell cycle progression through stimulation of cyclin expression and translation.[43,44] A role for

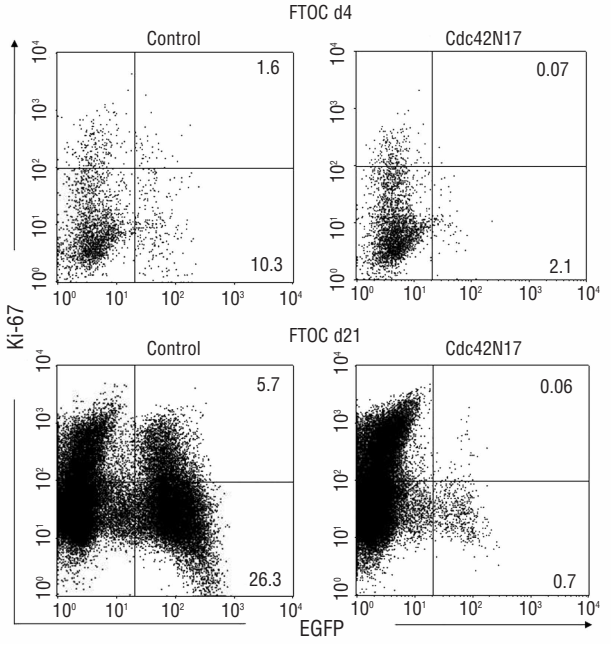

Figure 5. Impaired proliferation in thymocytes expressing dominant negative Cdc42N17 mutant. Representative flow cytometric analysis after 4 days (upper panels) and 21 days (lower panels) of fetal thymus organ culture started from CD34$^+$ cells. Plots show thymocytes stained intracellularly with anti-Ki-67-PE. The number indicates the percentage of cells in the corresponding quadrant. Quadrants are set according to isotype controls. Results shown are representative of at least three independent experiments.

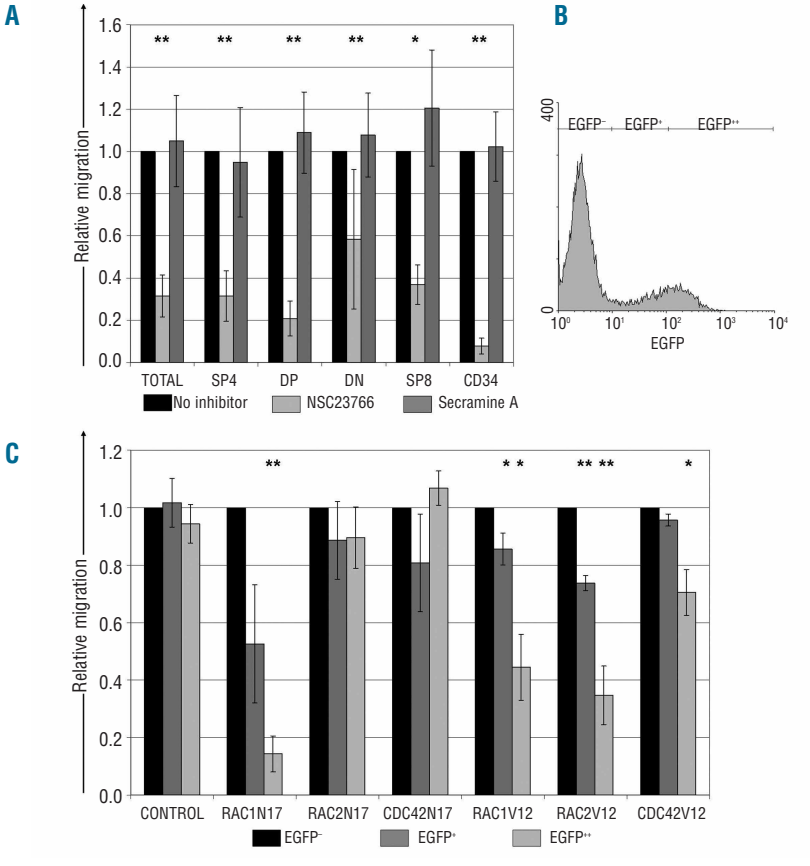

Figure 6. Chemotaxis of thymocyte subsets in response to SDF-1α. (A) The percentage of migrated cells in the presence of Rho GTPase inhibitors was calculated for each thymocyte subset. Relative migration is displayed, i.e. migration of a thymocyte subset in the presence of inhibitor normalized to migration of the same donor thymocyte subset in the absence of inhibitor. For total thymocytes (TOTAL), CD4$^+$CD8$^-$ (SP4), CD4$^+$CD8$^+$ (DP), CD4$^-$CD8$^-$ (DN) and CD4-CD8$^+$ (SP8) cell fractions, thymocytes migrated 'in bulk' and subsets were determined by labeling with monoclonal antibodies and subsequent flow cytometry. For CD34$^+$ cells (CD34), cells were sorted and analyzed in separate transwells. Asterisks indicate statistically significant differences between inhibition by NSC23766 and control (*P<0.05; **P<0.01). (B) Representative flow cytometric analysis of EGFP expression of CD34$^+$ cells transduced for chemotaxis assays. Gates EGFP$^-$ to EGFP^{++} indicate expression intervals used for the calculation of migration of transduced cells as shown in (C). (C) Migration of CD34$^+$ thymocytes expressing mutant Rho GTPases, normalized to non-transduced EGFP$^-$ cells in the same transwell. Asterisks indicate statistically significant differences between EFGP$^+$ or EGFP^{++} cells and EGFP$^-$ cells (*P<0.05; **P<0.01). (A,C) Bars indicate the mean of at least three independent experiments and error bars indicate standard deviation.

Cdc42 in proliferation seems to be highly cell type-specific, as conditional Cdc42$^{-/-}$ mouse hematopoietic stem cells display increased proliferation, while cell cycle progression of embryonic stem cells is unaffected and embryonic fibroblasts are reduced in the absence of Cdc42.[15,45,46] However, it has been shown that Cdc42 is activated upon TCR engagement of murine T cells, and expression of dominant negative Cdc42 results in reduced proliferation upon full T-cell activation.[47] It is, therefore, tempting to speculate that disturbance of proliferation (downstream of the pre-TCR) is at least in part responsible for the observed defects in T-cell development in our model. In line with this, apoptotic death, the default pathway for thymocytes not triggered to proliferate and mature, was enhanced in thymocytes expressing dominant negative Cdc42 compared to that occurring in control transduced cells. Together, these experiments reveal an important role for Cdc42 in proliferation and cell survival during normal T-cell development.

Transgenic mice expressing a constitutively active mutant of Cdc42 have reduced thymocyte numbers as a result of increased apoptosis in the thymus.[20] We showed that expression of a constitutively active mutant in human CD34^{+} progenitor cells also results in reduced thymocyte generation. Thus, Cdc42 activity must be kept in check to allow T-cell development.

The discrepancy between the inhibition of T-cell development mediated by expression of constitutively active Rac2, and the significant but minor effect of the dominant negative mutant of Rac2, is similar to that found in studies of transgenic and knock-out mice.[9,11,48] One explanation that should be considered, and which is supported by expression analysis, is that Rac2 is not abundantly active in the early stages of T-cell development. Enforced expression and activation might cause aberrant effects, not related to its true function. However, another possibility is that Rac1 activation partially compensates for loss of active Rac2 and *vice versa*. Double transduction experiments could have resolved this question, but were technically not possible because of the low frequency of double transduced cells. This hypothesis is, however, supported by mice studies comparing T-cell development in Rac1$^{-/-}$/Rac2$^{-/-}$ double knock-out mice and single knock-out mice.[9,10,12]

Expression of dominant negative Rac1 did not result in a lower, but rather a higher thymocyte generation ratio, as compared to control transduced cells, while migration was severely impaired. This suggests that Rac1-dependent migration to SDF-1α is not necessary for T-cell development in this model, or that residual migration is enough to provide the necessary signals to developing thymocytes. Among the dominant negative mutants, only Rac1N17 disturbs migration, while all constitutively active mutants impair migration to a certain extent. We conclude from our experiments that there is no correlation between thymocyte development and migration to SDF-1α after manipulation of Rho GTPase activity.

All constitutively active mutants disturb T-cell development and we noted that a high transgene expression level correlated with higher CD3 expression and higher TCR expression. It is unclear whether this skewing of CD3 was the result of a direct effect of Rho GTPases on CD3 expression, or an indirect developmental effect. Apart from this, we found no phenotypic differences between cells expressing these constitutively active mutants or EGFP only, most CD3^{+} cells being CD4^{+}CD8^{-}.

Dominant negative Rho GTPase mutants operate by sequestering guanosine nucleotide exchange factors away from the endogenous counterparts. The first implication is that these mutants need to be over-expressed in large excess in order to effectively inhibit activation of endogenous protein. Rac1N17 disturbs migration while Cdc42N17 hampers T-cell development. Hence, these functional effects, considered together with the results of the control experiments performed, convincingly demonstrate that the constructs are functional. Rac2N17 does not result in major alterations of cell behavior. We demonstrated that expression of the transgene effectively led to generation of the protein, detected by western blotting (*data not shown*). We cannot, however, exclude that the level of expression is too low to result in effective block of endogenous Rac2 activation.

A second implication of the use of dominant negative mutants is the concern about specificity, especially with regard to Rho GTPases from the same subgroup, such as Rac1 and Rac2, which share Rho guanosine nucleotide exchange factors.[49,50] To account for this, we applied two different methods to inhibit the function of Rho GTPases in chemotaxis, i.e. administration of inhibitors and gene transfer of dominant negative mutants. Given the duration of the fetal thymus organ culture and the short half-life of the inhibitors, inhibitors could not be used in this culture. Furthermore, inhibitors could affect thymic stroma besides the thymocytes. However, the differential outcome of both chemotaxis assays and fetal thymus organ culture upon expression of Rac1N17 and Rac2N17 argues for biological activity of these mutants. For example, we found that Rac1N17 blocked migration while Rac2N17 did not. If Rac2N17 reduces Rac1 activity, it should also block migration. However, we cannot exclude that Rac1N17 has a broader effect than competing only with guanosine nucleotide exchange factors specific for Rac1. Despite their very high overall amino acid sequence similarity, Rac1 and Rac2 can be targeted to different subcellular locations via the hypervariable C-terminus.[51] This could explain their specific functions, and it could also explain the rather specific action of the mutants although they can be activated by the same guanosine nucleotide exchange factors and activate mutual effectors.

In conclusion, we have demonstrated that Cdc42 is essential for T-cell development but not for migration of thymocytes towards SDF-1α. Increased apoptosis and reduced proliferation were found in thymocytes expressing dominant negative Cdc42. The role of Cdc42 in pathological development of human T cells and a possible Cdc42 deregulation in their malignant counterparts merit further research.

Authorship and Disclosures

KS and BV designed the study, coordinated the research and wrote the paper. KS, VI, VS, PVH, EN, PM, KA, MB and BV collected data and participated in the statistical analysis and interpretation. JP interpreted data. All authors revised and approved the final manuscript.

The authors reported no potential conflicts of interest.

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