Altered cellular immunity in transgenic mice with T cell-specific expression of human D4-guanine diphosphate-dissociation inhibitor (D4-GDI)

Kensuke Kondoh1,2,3, Yuji Nakata1,2,4, Takashi Yamaoka5, Mitsuo Itakura5, Mutsumi Hayashi1,2, Kohji Yamada1, Jun-ichi Hata1,6 and Taketo Yamada1

1Department of Pathology and 2Department of Pediatrics, Keio University School of Medicine, 35 Shinano-machi, Shinjuku-ku, Tokyo 160-8582, Japan
3Department of Pediatrics, St Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kanagawa 216-8511, Japan
4Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA
5Otsuka Department of Clinical and Molecular Nutrition, Tokushima University School of Medicine, Tokushima, Japan
6National Center for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan

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Abstract
D4-GDI, a Rho guanosine diphosphate (GDP) dissociation inhibitor, is preferentially expressed in hematopoietic tissues and binds to a small GTP-binding protein, Rho, and inhibits GDP dissociation from Rho. We identified point mutations in the D4-GDI gene in human leukemic cells. We therefore investigated the functions of D4-GDI and mutated D4-GDI in T cells. Transgenic mice (Tg) harboring human wild-type and mutant D4-GDI transgenes driven by the Ick promoter were generated. Cellular immunity responses against cytozoic pathogens were examined. The cytoskeletal organization in the CD3+ T cells and the proliferation of splenocytes by Con A were investigated in both Tg and littermates (LMs). Granuloma formation by bacille Calmette-Guerin was impaired in the wild-type D4-GDI Tg. On the other hand, the number of granulomas of the mutated D4-Tg was significantly higher. Infection with Listeria was more rapidly fatal to wild-type D4-GDI Tg than to LMs, while the survival of mutated D4-GDI Tg was prolonged. The CD3+ T cells in wild-type D4-GDI Tg showed an impairment in the formation of stress fibers on anti-CD3 antibody-coated plates, whereas the cytoskeletal organization in CD3+ T cells of the mutated D4-GDI Tg was augmented. The proliferation of splenocytes after Con A stimulation was higher in the mutated D4-GDI Tg than in the LMs. D4-GDI may have important functions, such as induction of T cell migration, adhesion and/or proliferation in inflammatory foci, in cellular immunity responses to cytozoic pathogens.

Introduction
Rho, Rac and Cdc42 GTPases belong to a family of small GTP-binding proteins that forms part of the Ras superfamily; these GTPases regulate various actin filament-dependent cell functions, such as cell adhesion, cell motility and cytokinesis (1, 2, 3, 4, 5), as well as the expression of certain genes (6). The GTPases are only active in a GTP-bound state and the exchange of GTP and guanosine diphosphate (GDP) is strictly regulated by three types of regulatory proteins: GDP dissociation stimulators (GDSs), GDP dissociation inhibitors (GDIs) and GTPase-activating proteins (GAPs). Some GDS proteins and GAPs from the Rho family and three Rho-GDIs have been isolated (7). D4-GDI, one of the Rho-GDIs, is preferentially expressed in hematopoietic cells, and Rho-GDI is expressed in the brain, lungs, kidneys, testes and pancreas, while Rho-GDI is ubiquitously expressed in all mammalian organs (8, 9, 10). Rho-GDI binds the majority of Rho family GTPases in the cytoplasm, maintaining Rho in an inactive form in which it cannot interact with effector targets or other regulatory proteins (11). On the other hand, Rho-GDI is also weakly associated with the GTP-bound forms of Rho, Rac and Cdc42 (12, 13). This weak interaction results in the inhibition of the intrinsic and GAP-stimulated GTPase activities of the Rho GTPases. Thus, Rho-GDI appears to be capable of blocking the GTP binding/GTPase cycle at two points: at the GDP/GTP exchange step and at the GTP hydrolytic step. Further studies...
have demonstrated that Rho-GDI associates with a Rho-GDI displacement factor from the ezrin/radixin/moesin (ERM) family. ERM interacts with both an adhesion molecule, CD44, and F-actin, resulting in the association of the actin cytoskeleton with the plasma membrane (14). D4-GDI has been identified as a Rho-GDI-like protein that is ~68% homologous with Rho-GDI and is preferentially expressed at very high levels in hematopoietic cells, including erythroid, granulocytic, monocytic and lymphoid cells (8). In another report, expression of D4-GDI in lymphocytes was emphasized and D4-GDI was named Ly-GDI (9). The inhibitory effect of D4-GDI on GDP dissociation was specific for Rho, but not for Ras or Rap (8). Stimulation of T lymphocytes and myelomonocytic cells with phorbol esters leads to phosphorylation of D4-GDI on serine/threonine residues, raising the possibility that D4-GDI is involved in signaling pathways in these cells (9, 15). In addition, D4-GDI is constitutively phosphorylated on tyrosine residues in neutrophils (16). It is not known if the function of D4-GDI in these cells is also regulated by extracellular signals. Recently, Groysman et al. (17, 18) reported that the distribution of D4-GDI is altered in stimulated T cells and accumulates with Vav1 in the membrane extensions in the periphery of the ‘immunological synapse’ and D4-GDI is specifically activated by TCR engagement and participates in transmitting extracellular signals in T cells. On the other hand, D4-GDI has been reported to be a substrate of the apoptosis protease CPP32. D4-GDI was rapidly truncated to a 23-kDa fragment in Jurkat cells according to kinetics that parallel the onset of CPP32. D4-GDI was rapidly truncated to a 23-kDa fragment reported to be a substrate of the apoptosis protease signals in T cells. On the other hand, D4-GDI has been implicated in the engagement and participates in transmitting extracellular signals and/or changes in cell fragmentation during leukocyte apoptosis.

We identified two point mutations in the D4-GDI gene in a human leukemic cell line, KM3. Recently, we reported the same two point mutations in the D4-GDI gene in a human leukemic cell line, Reh, and suggested that the mutated D4-GDI functions as a dominant-negative molecule against the wild-type D4-GDI and accelerates invasion via regulation of cytoskeletal machinery (21). In the present study, to explore the functions of D4-GDI and mutated D4-GDI in T cells, we generated transgenic mice (Tg) using human wild-type and mutant D4-GDI transgenes driven by the lck promoter and analyzed T cell functions in vitro and in vivo.

Materials and methods

Human leukemic cells and cell culture

Two acute lymphoblastic leukemia cell lines (KM3 and HPB-ALL) and three Burkitt lymphoma cell lines (Raji [American Type Culture Collection (ATCC), CCL-86], Ramos [ATCC, CRL-1923] and Daudi [ATCC, CCL-213]) were examined. The KM3 cell line was established from a 12-year-old boy during his second relapse of acute lymphoblastic leukemia (22). The Raji cell line was derived from Burkitt lymphoma. The HPB-ALL cell line was derived from a pediatric T cell leukemia (23). Cells were cultured in the presence of 5% CO2 at 37°C using RPMI 1640 medium supplemented with 10% fetal bovine serum. Normal human peripheral blood lymphocytes from healthy Japanese men were also examined with an informed consent.

Reverse transcriptase PCR and DNA sequencing

Total RNA was extracted from each sample (5–10 × 10^6 cells) using ISOGEN (Nippon Gene, Toyama, Japan). RNA was reverse transcribed into first-strand cDNA using a First-Strand cDNA Synthesis Kit (Amersham-Pharmacia Biotech, Buckinghamshire, UK). D4-GDI cDNA was isolated by PCR amplification from first-strand cDNA using the N-terminal primer (5’-TAAATAGATCAGAAGTACTGAA-3’) and the C-terminal primer (5’-AGATTCTTCCACAGGTGCA-3’). PCR was performed in 10 mM Tris–HCl, pH 9.0, 2.0 mM MgCl2, 50 mM KCl, 0.2 mM of each deoxynucleoside triphosphate and 0.5 µM of each PCR primer using Taq DNA Polymerase (Toyobo, Tokyo, Japan). Thirty cycles were run with denaturation at 94°C for 60 s, annealing at 55°C for 60 s and extension at 72°C for 60 s. Reverse transcriptase (RT)-PCR products were cloned into a pGEM-T vector (Promega, Madison, WI, USA) and analyzed with a Thermo Sequenase Fluorescent-Labeled Primer Cycle Sequencing Kit (Amersham-Pharmacia Biotech) using T7 and Sp6 fluorescent primer and a DNA Sequencer (MegaBase 1000, Molecular Dynamics, Sunnyvale, CA, USA). The fluorescent primers used for sequencing were forward, 5’-GTCCGAGGAAATGGACAAAGAT-3’ and reverse 5’-TCCAGTAAGGTCCATTGTGATT-3’.

Sequences of genomic D4-GDI DNA

Sequence was prepared from KM3 cells by standard methods with SDS–protease K (24). A portion of the D4-GDI gene that included the mutations was amplified using the N-terminal primer (5’-CACCACAGAAGTCCCTGAAAGA-3’) and the C-terminal primer (5’-TCCAGTAAGGTCCATTGTGATT-3’). PCR products were cloned into a pGEM-T vector and sequenced. After partial sequencing of the D4-GDI intron (data not shown), PCR products were analyzed by direct sequencing methods using the fluorescent forward primer (5’-CACCACACTATACACATGTCTCT-3’) for the D4-GDI gene intron. KM3 cells were also obtained from other laboratories and the D4-GDI gene was sequenced by the following method in order to eliminate any contamination of cells and confirm the mutations. RT-PCR was performed with another N-terminal primer (5’-ACAGAGAGCTGAAGCAGTCTGAA-3’) and C-terminal primer (5’-GATGCAATAAGGAAATGTT-3’). These primers flanked the initial primers and were used to exclude contamination of PCR products and plasmids. PCR products were analyzed by the direct sequencing method.

Transgenic mice

Human wild-type and mutated D4-GDI cDNA, cloned from HPB-ALL and KM3 cells, respectively, were ligated into the expression vector, plick-hGH (provided by W. Henning, Imperial Cancer Research Foundation, London University). A 6.0-kb NotI fragment was microinjected into the
pronucleus of (C57BL/6J × DBA2) F1 fertilized ovum. The genomic DNA of two founder lines containing the wild-type D4-GDI gene and two founder lines containing the mutated D4-GDI gene was screened by PCR. Tg and the littermates (LMs) were generated by backcrossing with C57BL/6J mice (Clea, Tokyo, Japan). Transgene-carrying mice were identified by PCR of the genomic DNA purified from tail specimens. The following primers were used: Lck-1 (5′-CACGAAAGTGGTAACCTAGCTA-3′) and D4-c (5′-TCCAGTAAGGTCCATGTGATT-3′). Stable lines were generated by backcrossing the transgene-carrying founder mice with C57BL6/J mice. Adult female mice (5–10 weeks old) were then used in the analyses detailed below.

**Cell preparation**
The dissected thymi and spleens were minced with fine forceps and forced through a fine mesh filter to obtain a single-cell suspension. Thymocytes and splenocytes were additionally subjected to hypotonic shock to lyse the RBCs. The total cell numbers were determined by microscopic observation of the cells using a Neubauer hemocytometer.

**Western blot analysis**
The expression of D4-GDI protein was confirmed by Western blotting using a rabbit anti-D4-GDI polyclonal antibody (Zymed Laboratory, San Francisco, CA, USA) and an anti-c-Myc mAb (clone 9E10; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blotted membranes were treated with peroxidase-conjugated anti-rabbit Ig antibody and visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Affinity precipitation of cellular GTP-bound Rho and Rac**
Ren et al. (25) developed a method based on evidence that Rho effectors only interact with GTP-bound Rho to measure Rho activity (25). The binding of Rho to the Rho-binding domain (RBD) from the effector protein Rhotekin inhibited both the intrinsic and the GAP-enhanced GTPase activity of Rho (26). Cells were washed with ice-cold Tris-buffered saline and lysed in RIPA buffer [50 mM Tris, pH 7.2; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS; 500 mM NaCl; 10 mM MgCl2; 10 mg ml−1 each of leupeptin and aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Cell lysates were separated by centrifugation at 13 000 × g at 4°C for 10 min, and equal volumes of lysates were incubated with glutathione S-transferase (GST)-RBD (a fusion of RBD with GST, 20 μg) beads at 4°C for 45 min. The beads were washed four times with buffer B (Tris buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM MgCl2, 10 mg ml−1 each of leupeptin and aprotinin and 0.1 mM PMSF). Bound Rho proteins were detected by western blotting using an anti-RhoA mAb (Santa Cruz Biotechnology). A densitometric analysis was performed using NIH Image, Version 1.62.

**Flow cytometry analysis**
Antibodies (Becton Dickinson PharMingen, Mountain View, CA, USA) were conjugated to either FITC or PE. Thymocytes and splenocytes were immunohistochemically stained to detect the surface expression of CD4, CD8, CD25, CD44, CD2, αβTCR and B220 using appropriate antibodies.

**Apoptosis of splenocytes**
Freshly isolated spleen cells were used for dexamethasone- and ionomycin-induced cell death assays. Cells were cultured in the presence of 10 μM dexamethasone or 5 μM ml−1 ionomycin for 19 h and then stained with annexin V and propidium iodide (PI). For the TCR-induced apoptosis assays, the spleen lymphocytes were stimulated with Con A (5 μM ml−1) for 3 days and then subsequently cultured in recombinant human IL-2 (100 U ml−1) for 2 days, as described previously (27). The activated T cells were then cultured at 5 × 106 cells per well in flat-bottom 96-well plates that had been coated with anti-CD3ε antibody (2C11). After the desired culture times, the cells were harvested, stained with annexin V and PI and analyzed by flow cytometry (EPICS XL: Beckman Coulter, Hialeah, FL, USA) and dead cells were quantified as described previously (27).

**Nuclear translocation of D4-GDI following cleavage by caspase-3**
Freshly isolated thymocytes were used for a dexamethasone-induced cell death assay. Cells were cultured in the presence of 0.1 mM dexamethasone for 4 h. The cells were then pelleted, and the medium was removed. The cells were re-suspended in a hypotonic solution [50 mM HEPES, pH 7.0; 250 mM sucrose; 2 mM N-ethylmaleimide; 1 μg ml−1 pepstatin A; and 1 mM 1-(aminocetyl)-benzenesulfonyl fluoride hydrochloride], incubated on ice for 15 min, transferred to a homogenizer and lysed with 25 strokes. The lysate was then centrifuged for 10 min at 700 × g, and the pellet was re-suspended in 1 ml of 0.5 M sucrose in HSSE (50 mM HEPES, pH 7.0; 0.75 mM spermidine; 0.15 mM spermine; 0.1 mM EDTA). To further purify the nuclei, the re-suspended pellet was gently layered over 1.5 M of sucrose in HSSE, and the nuclei were pelleted in a microcentrifuge at 13 000 r.p.m. for 10 min. The supernatant from the 700 × g spin was centrifuged at 100 000 × g for 1 h. The resulting pellet consisted of light and heavy membrane fractions, with the supernatant containing the cytoplasmic fraction. The nuclei and cytoplasmic fractions were solubilized with 2% SDS buffer and subjected to a western analysis using a rabbit anti-D4-GDI polyclonal antibody (Zymed Laboratory). A densitometric analysis was performed using NIH Image, Version 1.62.

**Bacille Calmette-Guerin infection assays**
**Mycobacterium bovis** Bacille Calmette-Guerin (BCG) was obtained from Nihon BCG (Tokyo, Japan). Mice were immunized against BCG made by the intracutaneous injection of
with 0.5 mg of BCG. Fourteen days after the intracutaneous injection of BCG, the mice were infected intravenously with 0.05 mg of BCG. After 7 days, the mice were sacrificed, and formaldehyde-fixed, hematoxylin- and eosin-stained liver sections were used to quantify the granulomas. CD4+ cells and CD8+ cells in the granulomas were immunohistochemically stained using anti-CD4 and anti-CD8 antibodies (PharMingen), respectively. The in situ hybridization of mouse IFN-γ was immunohistochemically visualized using digoxigenin-labeled single-strand RNA probes for IFN-γ prepared using a DIG RNA Labeling Kit (Boehringer Mannheim GmbH Biochemica, Manheim, Germany), according to the manufacturer’s instructions. A 0.237-kb fragment of mouse IFN-γ cDNA was obtained by reverse transcription, followed by RT-PCR and then sub-cloned into Bluescript I pKS (−). BCG-immunized mice were infected intravenously with 0.05 mg of BCG. Three days later, the serum concentrations of IFN-γ were analyzed using an ELISA. BCG-immunized mice were also infected intravenously with 0.8 mg of BCG. After 7 days, the mice were sacrificed, and serial dilutions of whole spleen homogenates were plated on Kudo-PD culture plates (Nihon BCG supply). The number of bacterial colonies that had formed after 21 days of incubation (37°C, 5% CO₂) was then counted.

*Listeria monocytogenes* infection assays

*Listeria monocytogenes* was grown in tryptic soy broth (Difco Laboratories, Detroit, MI, USA) at 37°C for 16 h, washed repeatedly, suspended in PBS and stored at −70°C. C57/BL6/J mice were intravenously infected with graded doses of *L. monocytogenes*, and the number of deaths was recorded daily. After 14 days of observation, the mortality rate was calculated for each group of five to six mice that had received a given dose, and the median lethal dose (LD50) was determined. Mice were then intravenously infected with two doses of the already determined LD50 of *L. monocytogenes*, and the number of deaths was recorded daily.

**Intracellular cytokine assay for spleno-CD3+ cells**

Splenocytes were suspended in 10% FCS + RPMI 1640 containing 10 μg ml⁻¹ of Brefeldin A (Golgi Plug) to stop the transport of the cytokine products in the Golgi apparatus. The cells were then incubated with 40 ng ml⁻¹ of phorbol 12-myristate 13-acetate (PMA) (Sigma) and 4 μg ml⁻¹ of ionomycin (Sigma) in 5% CO₂ at 37°C. After 4 h of incubation, the cells were harvested and used in staining experiments. For three-color staining, the cells were incubated with CD3 Cy-Chrome in the dark at 4°C for 20 min. The cells were then washed once with 2 ml of Staining Buffer (PharMingen) and spun at 400 × g at 4°C for 5 min. The cells were then fixed and permeabilized using 100 ml of Cytofix/Cytoperm for 20 min at 4°C. The cells were washed once (using 1× Perm/Wash, 400 × g, 5 min, 4°C) and then incubated with FITC-conjugated anti-murine cytokine antibodies; IFN-γ (PharMingen) and PE-conjugated anti-murine cytokine antibodies: IL-4 (PharMingen) for 30 min at 4°C in the dark. The cells were then washed twice for 15 min at 4°C with 1× PermWash and were re-suspended in 0.5 ml of Staining Buffer. The cells were then analyzed by flow cytometry (EPICS XL-MCL, Beckman Coulter).

**Capping studies**

Splenocytes at a plate concentration of 3 × 10⁷ cells ml⁻¹ were stimulated for 90 min with 25 μg ml⁻¹ plate-bound anti-CD3ε antibody (2C11). The cells were fixed, and F-actin was visualized by staining with fluorescein phallolidin (Molecular probes, Eugene, OR, USA). The cells were then analyzed using immunofluorescence microscopy. The splenocytes stimulated with anti-CD3ε antibody (2C11) were stained with fluorescein phallolidin and analyzed by flow cytometry (EPICS XL, Beckman Coulter). In addition, the cellular GTP-bound RhoA in splenocytes that had been stimulated with anti-CD3ε antibody (2C11) for 120 min were immunoprecipitated, and densitometric analysis was performed using NIH Image, Version 1.62.

**Labeling of spleen cells with carboxyfluorescein diacetate succinimidyl ester and analysis of T cell division**

Spleen cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) as described by Parish et al. (28). Briefly, splenocytes were suspended in PBS at a concentration of 5 × 10⁶ ml⁻¹, and CFSE was added to the cells at a final concentration of 5 μM. After incubation at 37°C for 10 min, the cells were washed with RPMI 1640 culture medium and incubated on ice for 5 min to terminate the reaction. Cells stained with CFSE were stimulated with 5 μM of Con A. A cytometric analysis of T cell division was then performed.

**Results**

**Detection of mutations of D4-GDI cDNA or genomic DNA in human leukemic cells**

The results of DNA sequence analysis of the D4-GDI cDNA from the human leukemic cell lines are shown in Fig. 1(A). Two point mutations at positions 276 (G to C change) and 280 (T to C) were found in the D4-GDI cDNA of the KM3 cell line. No mutations of the D4-GDI gene were detected in the human leukemic cell line. No mutations of the D4-GDI gene were detected in the D4-GDI gene sequences of wild-type D4-GDI and other Rho-GDI family genes show that these two mutations of D4-GDI exist in the partially conserved region at the amino acid level.

**Gene transfer of wild-type or mutated D4-GDI expression vector into mice**

To express human D4-GDI in only T cells, we used a transgenic expression vector based on the proximal promoter of p56^ck^ (Fig. 1A). To confirm that the transgene construct was expressed in the T cells of wild-type and mutated Tg, we performed western blotting analysis of the thymocytes using anti-D4-GDI polyclonal antibody. Thymocyte lysates...
from D4-GDI Tg yielded a transgene band corresponding to
myc-D4-GDI. In contrast, thymocyte lysates from LMs did
not show any transgene band (Fig. 1B). We also detected
the expression of exogenous D4-GDI protein in the spleno-
cytes using western blotting and an anti-c-Myc antibody
(9E10). Splenocyte lysates from D4-GDI Tg yielded a trans-
gene band corresponding to myc-D4-GDI. In contrast, thy-
mocyte lysates from LM did not show any transgene band
(Fig. 1C). The thymocytes and splenocytes of wild-type and
mutated D4-GDI Tg mice expressed a protein
\[30\text{kDa}\] that was recognized by the anti-D4-GDI antibody and
the anti-c-Myc antibody (9E10), respectively.

**Fig. 1.** Generation of the D4-GDI Tg. (A) Two point mutations of D4-GDI in KM3 cells and alignment of the predicted amino acid sequences of
mutated D4-GDI and wild-type D4-GDI are shown (upper panel). Two point mutations in D4-GDI were detected in KM3 cells. These
changes resulted in a guanine to cytosine substitution at nucleotide 276 and a thymine to cytosine substitution at nucleotide 280. The two D4-GDI
point mutations in KM3 cells led to a valine to leucine change at position 68 and a valine to alanine change at position 69 (underlined). Schema of
the D4-GDI transgene construct (lower panel). Expression of wild-type and mutated D4-GDI is also driven by the proximal p56lck promoter, restricting expression of the transgene to T cells. myc, myc-tag; hGH, 625 bp of the human growth hormone gene 3’ untranslated region
sequence encoding a consensus polyadenylation site. (B) Western blotting of thymocytes was performed using anti-D4-GDI polyclonal antibody, peroxidase-conjugated secondary antibody and electrochemiluminescence for visualization. Lane 1: HPB-ALL (a positive control for endogenous D4-GDI; arrowhead), lane 2: Raji cells transfected with the myc-D4-GDI transgene (positive control for the transgene), lane 3:
LM, lane 4: LM, lane 5: wild-type Tg line A, lane 6: wild-type Tg line B, lane 7: wild-type Tg line C, lane 8: wild-type Tg line D (left panel). Lane 1:
KM3-ALL (a positive control for endogenous mutated D4-GDI), lane 2: Raji cells transfected with the myc-mutated-D4-GDI transgene (positive
control for the transgene), lane 3: LM, lane 4: LM, lane 5: mutated Tg line S, lane 6: mutated Tg line T, lane 7: mutated Tg line V, lane 8: mutated
Tg line W (right panel). The thymocytes of wild-type and mutated D4-GDI Tg expressed proteins of \[\sim 30\text{kDa}\]. The 30-kDa bands were exogenous
D4-GDI with a myc-tag (arrow). (C) Western blotting of splenocytes was performed using anti-Myc-tag mAb, peroxidase-conjugated secondary
antibody and electrochemiluminescence for visualization. Lane 1: wild-type D4-GDI Tg line C, lane 2: LM, lane 3: mutated D4-GDI Tg line T,
lane 4: LM. The 30-kDa bands were exogenous D4-GDI with a myc-tag (arrow).

**Altered immunity in D4-GDI transgenic mice**

from D4-GDI Tg had a normal number of leukocytes in their peripheral blood (data not shown). Both wild-type and mutated D4-GDI Tg
had a normal number of leukocytes in their peripheral blood (data not shown). The percentages of the various lymphocyte subsets in freshly isolated thymus, spleen and lymph
node were examined using flow cytometry. Cells derived from the spleen and thymus of wild-type and mutated
D4-GDI Tg exhibited a normal surface expression of CD4 and CD8 (data not shown). Further analyses of spleen and lymph node tissues revealed that a variety of other markers,
including CD3, CD2, CD25, CD28, CD45, B220 and Mac1, were unaffected by the over-expression of wild-type and mutated D4-GDI (data not shown). Together, these data
suggest that development, in general, and lymphoid differentiation, in particular, are apparently normal in wild-type
and mutated D4-GDI Tg.

**Detection of cellular GTP-bound RhoA and Rac1 was unaltered in resting thymocytes**

We next investigated whether D4-GDI (wild-type or mutated)
over-expression in thymocytes altered Rho and Rac1 activity.
We employed a pull-down assay using RBD and PAK affinity precipitation and western blotting with anti-RhoA and anti-Rac1 antibodies, respectively. The proportions of activated RhoA and Rac1 (GTP-bound RhoA or Rac1/total RhoA or Rac1) did not differ among wild-type or mutated D4-GDI Tg and the LMs (Fig. 2A and B). The GTP-bound RhoA and Rac1 accounted for 1.3–2.0% and 0.8–1.1% of the total RhoA and Rac1, respectively, in all experiments. These data suggest that the over-expression of wild-type and mutated D4-GDI did not affect the activity of RhoA and Rac1 in resting T cells.

Apoptosis in T cells from wild-type and mutated D4-GDI Tg does not differ from that in T cells from LMs
To determine whether the over-expression of wild-type and mutated D4-GDI affects the apoptosis of T lymphocytes, several inducers of apoptosis were examined: dexamethasone or ionomycin, TCR stimulation and IL-2 withdrawal. Programmed cell death was not globally different between wild-type D4-GDI Tg and LMs or the mutated D4-GDI Tg and LMs (Fig. 3A–D). D4-GDI has been reported to be cleaved by caspase-3 during apoptosis, and caspase cleavage triggering translocation of a substrate from the cytoplasm to the nucleus of cells (20). To determine whether mutation of D4-GDI affects the cleavage and translocation of D4-GDI, we performed western analysis of the cytoplasmic and nuclear fractions of thymocytes using a rabbit anti-D4-GDI polyclonal antibody, after inducing apoptosis with dexamethasone. When compared with wild-type D4-GDI; however, the cleavage and translocation of mutated D4-GDI was not different (Fig. 3E).

Immune response of wild-type and mutated D4-GDI Tg infected with BCG or L. monocytogenes differs from that in infected LMs
To determine whether the over-expression of wild-type or mutated D4-GDI in T cells influenced the immune response to infection with intracellular bacteria, we performed infection assays using BCG or L. monocytogenes. In the BCG infection assay, BCG-immunized mice produced granulomas in their livers 7 days after the injection of BCG (Fig. 4A). Within the granulomas, the CD4-positive cells formed clusters. The hepatic granulomas of wild-type D4-GDI Tg were smaller than those of LMs, and the number of granulomas produced was significantly smaller. On the other hand, the hepatic granulomas of the mutated D4-GDI Tg were larger than those of LMs, and the number of granulomas in the mutated D4-GDI Tg was significantly higher (Fig. 4B and C). The clusters of CD4+ cells and IFN-γ-positive cells in wild-type D4-GDI Tg were smaller than those of LMs, while the clusters of CD4-positive cells and IFN-γ-positive cells in the mutated D4-GDI Tg were larger than those of LMs (Fig. 4D and E). However, compared with LMs, the serum concentration of IFN-γ in the wild-type D4-GDI Tg was not altered, while the serum concentration of IFN-γ in the mutated D4-GDI Tg was significantly lower (data not shown). The bacterial burden at 7 days after the infection of BCG in BCG-immunized mice was compared counting the colony-forming units (CFU) recovered from the spleen. When

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**Fig. 2.** Detection of cellular GTP-bound Rho and Rac in the thymocytes of Tg. (A) Cell lysates were clarified by centrifugation, and equal volumes of lysates were incubated with 20 µg GST-RBD beads. The beads were washed four times, and bound Rho proteins were detected by western blotting using an anti-RhoA mAb. The upper figures show the expression of GTP-bound Rho and total Rho. In order to quantify the amount of GTP-bound Rho, densitometric analysis was performed using NIH Image, Version 1.62. The amount of RBD-bound Rho was normalized to the total amount of Rho in cell lysates for comparison of Rho activities (level of GTP-bound Rho) among different samples. The ratio of GTP-bound Rho to total Rho is shown in the lower graph. (B) Measurement of Rac activity was performed using a Rac Activation Assay Kit (Cytoskeleton) in accordance with the manufacturer’s protocol. The upper figures show the expression of GTP-bound Rac and total Rac. In order to quantify the amount of GTP-bound Rac, densitometric analysis was performed using NIH Image, Version 1.62. The amount of GTP-bound Rac was normalized to the total amount of Rac in cell lysates for comparison of Rac activities (level of GTP-bound Rac) among different samples. The ratios of GTP-bound Rac to total Rac are shown in the lower graph. These figures show one representative experiment among three performed, respectively.
compared with the LMs, the number of CFU recovered from the spleens of the wild-type D4-GDI Tg was higher, while the number of CFU recovered from the spleens of the mutated D4-GDI Tg was lower (Fig. 4F).

In the *L. monocytogenes* infection assay, we performed a challenge test by inoculating mice with two doses of the LD50 of *L. monocytogenes*. The wild-type D4-GDI Tg died more quickly than LMs after inoculation with *L. monocytogenes*; however, the other mutated D4-GDI Tg showed significantly longer survival (Fig. 5).

**No alteration of intracellular cytokine levels of spleno-CD3+ cells stimulated by PMA and ionomycin**

To determine whether the over-expression of wild-type or mutated D4-GDI in T cells affects the Th1/Th2 ratio following activation by short-term PMA and ionomycin, we analyzed the cells using triple-color flow cytometry (CD3, IFN-γ and IL-4) (Table 1). The ratio of Tn1/Tn2 in CD3+ splenocytes from wild-type and mutated D4-GDI Tg and LMs were not globally different.

**Actin cytoskeletal changes are suppressed in wild-type D4-GDI T cells but enhanced in mutated D4-GDI T cells**

First, to determine whether over-expression of wild-type or mutated D4-GDI in T cells affects the cytoskeleton in response to TCR signaling, we examined whether such over-expression altered actin morphology following antigen receptor signaling. After anti-CD3ε antibody cross-linking, new clusters or ‘patches’ of actin were visualized by fluorescein phalloidin staining in mature T lymphocytes. Compared with LMs, this activation-dependent change in F-actin morphology was suppressed in wild-type D4-GDI T cells and enhanced in mutated D4-GDI Tg (Fig. 6A). Second, we analyzed this fluorescein phalloidin staining in mature T lymphocytes using flow cytometry. The intensity of F-actin expression was enhanced only in mutated D4-GDI Tg (Fig. 6B). Finally, we analyzed the RhoA activity in wild-type...
or mutated D4-GDI splenocytes with or without anti-CD3e antibody (2C11) stimulation. We found that RhoA activity in wild-type D4-GDI splenocytes without stimulation by plate-bound anti-CD3e antibody (2C11) was decreased, whereas that of mutated but unstimulated D4-GDI splenocytes was increased. However, there was no significant difference of RhoA activity between stimulated wild-type and stimulated mutated D4-GDI splenocytes (Fig. 6C).

Fig. 4. BCG infection assay. (A) Experimental design of BCG infection assays. (B) Hepatic granulomas of BCG-infected BCG-immunized D4-GDI Tg or LM mice (hematoxylin and eosin stain). (C) The number of hepatic granulomas in BCG-infected, BCG-immunized D4-GDI Tg or LM mice. Shaded and clear squares represent cells from D4-GDI Tg and control LM mice, respectively. The results are an average of three independent experiments. (D) Existence of CD4+ cells in hepatic granulomas of the BCG-infected BCG-immunized D4-GDI Tg or LM mice. (E). Existence of infiltrated cells expressing IFN-γ in the granulomas of BCG-infected, BCG-immunized D4-GDI Tg or LM mice (in situ hybridization for IFN-γ). (F) Numbers of CFU BCG recoverable from spleens of BCG-infected, BCG-immunized D4-GDI Tg or LM mice. Shaded and clear squares represent cells from D4-GDI Tg and control LM mice, respectively. These figures show one representative experiment among three performed, respectively.
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Fig. 5. Survival after infection with *Listeria monocytogenes*. Kaplan–Meier survival analysis of D4-GDI Tg and LMs was performed after the infection with two doses of the already determined LD50 of *L. monocytogenes*.

Table 1. Intracellular cytokine assay of CD3+ splenocytes stimulated with PMA and ionomycin

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<thead>
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<th>Experiment 1</th>
<th>Experiment 2</th>
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<tr>
<td>wt LM</td>
<td>4.40</td>
<td>4.11</td>
<td>3.13</td>
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<tr>
<td>wt Tg</td>
<td>4.16</td>
<td>4.80</td>
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<tr>
<td>mt LM</td>
<td>5.11</td>
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<td>mt Tg</td>
<td>5.42</td>
<td>3.45</td>
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Ratio of T*eff1/T*eff2 in CD3+ splenocytes subjected to short-term stimulation with PMA and ionomycin was analyzed by triple-color flow cytometry (CD3, IFN-γ and IL-4).

**In vitro Con A-induced proliferation of splenocytes is enhanced in mutated D4-GDI**

To directly analyze the proliferation capacity of T cells from wild-type or mutated D4-GDI Tg T cells, single-cell suspensions of splenocytes were stained with CFSE and stimulated with Con A. The splenocytes from wild-type D4-GDI Tg showed no difference in proliferation compared with LMs. On the other hand, the splenocytes from mutated D4-GDI Tg showed higher proliferation activity compared with LMs (Fig. 7).

**Discussion**

D4-GDI is a highly abundant cytoplasmic protein found in lymphocytes that has been highly conserved since the divergence of mammalian species. It has been reported that Rho-GDI forms a complex with RhoA, Cdc42 and Rac, while Cdc42 and Rac were not found to interact with D4-GDI (15). Furthermore, stimulation of T lymphocytes and myelomonocytic cells with phorbol esters leads to phosphorylation of D4-GDI on serine/threonine residues, raising the possibility that D4-GDI is involved in signaling pathways in these cells (9, 15). In addition, D4-GDI is constitutively phosphorylated on tyrosine residues in neutrophils (16). These results suggest that D4-GDI can regulate specific signal pathways in hematopoietic cells.

However, D4-GDI knockout mice are viable and exhibit normal thymocyte selection and immune responses, regardless of decreased apoptosis of lymph node cells after IL-2 withdrawal (29). The normal development and regulation of D4-GDI null mice may be attributable to compensations for the defect by other GDIs, such as Rho-GDI, or other regulators of Rho, such as Rho-GAPs. Recently, Ishizaki et al. (30) reported that combined disruption of both the Rho-GDI and D4-GDI genes in mice resulted in reduction of marginal zone B cells in the spleen and retention of mature T cells in the thymic medulla. Furthermore, these mice showed lower CD3 expression. This would explain why D4-GDI null mice are viable and exhibit normal thymocyte selection and immune responses, i.e. through compensation of Rho-GDI.

We identified two point mutations of D4-GDI in a human B cell leukemic cell line. The region of D4-GDI containing these point mutations, which result in amino acid substitutions, is highly conserved among Rho-GDI family genes. X-ray analysis of the three-dimensional structure of Rho-GDI suggested that these mutations of D4-GDI are present in a region that folds into a β-sheet structure (31). This region is at the back of the continuous surface adjacent to the isoprene-binding site of Rho-GDI and may easily be in contact with the bound GTPase, imparting GDI activity. A protein secondary structure prediction by Robson suggested that these mutations may disrupt the β-sheet region (32). Furthermore, we have already demonstrated by (3H)GDP dissociation assay that a similar mutated form of D4-GDI functions in a dominant-negative manner in vitro (21).

Accordingly, we speculated that this mutated D4-GDI may also function in a dominant-negative manner with regard to other GDIs, such as Rho-GDI, and therefore, we generated Tg over-expressing the wild-type or mutated human D4-GDI gene in T cells via the lck promoter. However, the resting thymocyte selection in cells over-expressing wild-type D4-GDI or mutated D4-GDI Tg was normal, and the amount of GTP-bound Rho in resting thymocytes in cells over-expressing wild-type D4-GDI or mutated D4-GDI did not show any change in Rho activity. Furthermore, we speculated that the use of resting thymocytes in this assay was the reason why changes were not observed in these data. We also thought that D4-GDI may only function under specific conditions, such as apoptosis and during cellular immunity responses.

D4-GDI has been shown to be specifically cleaved at two positions (residues 18–19 and 54–55) by two different apoptosis proteases, caspase-3 and caspase-1, respectively (33, 34). These consensus cleavage sequences are not present in either Rho-GDI or Rho-GDI. A truncated D4-GDI cleaved by caspase-1 is unable to effectively bind and regulate Rho family members. D4-GDI is a target protein of caspase-3 in the processes of anti-IgM-mediated or Fas-dependent apoptosis (33, 35). Krieser et al. (20) have reported that the cleaved fragment of D4-GDI produced by caspase-3 is translocated to the nucleus. Furthermore, in serial studies on the role of Rho GTPases in regulation of apoptosis, Zhang et al. (36–39) have shown that D4-GDI is expressed in human breast cancer cells and modulates breast cancer cell invasive activities. We first observed that apoptosis in T cells over-expressing wild-type D4-GDI or mutated D4-GDI did not differ from that in T cells obtained from
LMs and that apoptosis was induced by irradiation, dexamethasone, ionomycin, TCR stimulation and IL-2 withdrawal. Second, we analyzed the ratio of cleaved D4-GDI and the ratio of translocated D4-GDI in thymocytes undergoing dexamethasone-induced apoptosis. We confirmed that the ratio of cleaved mutated D4-GDI and the ratio of translocated mutated D4-GDI did not differ from those of wild-type D4-GDI. The similarity of the apoptosis assay results can probably be explained by the positions of the point mutations, which affect residues 68 and 69. Together with the result of the apoptosis assay in D4-GDI knockout mice induced by IL-2 withdrawal, which showed partial inhibition of apoptosis, we thought that D4-GDI affects apoptosis under special conditions, such as activation-induced cell death. Activation-induced cell death has been reported to depend on interactions between ligands and receptors.

Fig. 6. Determination of cap formation using anti-CD3ε antibody. (A) Anti-CD3 antibody (2C11)-induced cap formation in splenocytes examined using F-actin staining. (B) Anti-CD3 antibody (2C11)-induced cap formation in splenocytes examined using flow cytometry. (C) Western blot analysis of affinity-precipitated cellular GTP-bound RhoA (top) and total RhoA (middle) in splenocytes of Tg was examined with (+) or without (−) stimulation with plate-bound anti-CD3ε antibody (2C11). Signal intensity was quantified using NIH Image, Version 1.62 (bottom). Gray and black squares represent cells from wild-type and mutated D4-GDI Tg, respectively. These figures show one representative experiment among three performed, respectively.

Fig. 7. In vitro Con A-induced proliferation of splenocytes. Splenocytes stained with CFSE were stimulated with 5 μM Con A, and cytometric analysis of T cell division was performed. These figures show one representative experiment among three performed, respectively.
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belonging to the tumor necrosis factor (TNF) family, which includes TNF and Fas, and on the susceptibility of the cells to receptor-mediated death signal transduction (40). These death-triggering pathways are involved in the elimination of antigen-stimulated peripheral T cells to terminate an immune response and to limit inflammation.

Next, we used BCG or L. monocytogenes infection assays to analyze cellular immune systems, since BCG and L. monocytogenes are facultative intracellular bacteria. Susceptibility to infection with BCG or L. monocytogenes was higher in wild-type D4-GD1 Tg and lower in mutated D4-GD1 Tg, compared with LMs. Especially in the BCG infection assay, the size and number of hepatic granulomas and the number of CD4+ and IFN-γ-positive cells were smaller/lower in wild-type D4-GD1 Tg, but were larger/higher in mutated D4-GD1 Tg, compared with LMs. However, in a short-term induction of NFAT activation, phospholipase C was similar in wild-type and mutated D4-GD1 Tg compared with these values in LMs. Over-expression of wild-type D4-GDI in T cells inhibited cellular immunity against BCG or L. monocytogenes. This impaired cellular immunity may be explained by an inhibitory effect of D4-GDI over-expression on the nuclear factor of activated T cells (NFAT). Groysman et al. (18) reported that over-expression of D4-GDI alone is inhibitory to NFAT stimulation and calcium mobilization but that when co-expressed with Vav1, D4-GDI enhances Vav1 induction of NFAT activation, phospholipase Cγ phosphorylation and calcium mobilization. This investigation suggests that the balanced distribution of both Vav1 and D4-GDI is altered in stimulated T cells.

Additionally, Rho-GDI may play a role in cancer invasion and metastasis via involvement in the CD44 signaling pathway, since Rho-GDI co-immunoprecipitated with the CD44-ERM complex (41). These findings obviously indicate that the Rho family and its regulatory proteins play critical roles in migration and adhesion. Based on these results, we speculated that D4-GDI affects the motility of T cells toward inflammation foci, explaining the differences in the susceptibility to infections with BCG and L. monocytogenes between wild-type and mutated D4-GDI Tg. To confirm this speculation, we performed a capping study by activating T cells with anti-CD3e antibody. Compared with LMs, the activation-dependent change in F-actin morphology was suppressed in wild-type D4-GDI T cells and enhanced in mutated D4-GDI T cells. To analyze these changes in F-actin morphology, we examined the total amount of F-actin in these cells using flow cytometry. We demonstrated no alteration in the content of F-actin in the wild-type D4-GDI T cells and found that the amount of F-actin in mutated D4-GDI T cells was increased compared with T lymphocytes in LMs. We speculated that the change of F-actin morphology in wild-type D4-GDI T cells was due to distribution of intracellular F-actin. In addition, we analyzed affinity precipitation of cellular GTP-bound RhoA in wild-type D4-GDI splenocytes with or without stimulation by plate-bound anti-CD3e antibody (2C11). We demonstrated that RhoA activity was increased in stimulated wild-type D4-GDI splenocytes but was already up-regulated to the stimulated level in unstimulated mutated D4-GDI splenocytes. These results suggested that the suppression of F-actin morphology in wild-type D4-GDI T cells was due to a decrease of non-specific cell contact through a decrease of RhoA activity and that the enhancement of F-actin morphology in mutated D4-GDI T cells was due to both an increase of non-specific cell contact through an increase of RhoA activity and an increase in the amount of F-actin through stimulation with plate-bound anti-CD3e antibody (2C11). In particular, the increase of F-actin in mutated D4-GDI T cells resulting from stimulation with anti-CD3e antibody supports the contention that D4-GDI is the molecule that mediates TCR stimulation. Groysman et al. (18) reported that Vav1 and D4-GDI, but not Rho-GDI, translocate to membrane extensions in contact with the antigen-presenting cell in response to superantigen stimulation. Furthermore, D4-GDI is tyrosine phosphorylated upon TCR stimulation and it associates with the SH2 region of the adapter protein Shc in a TCR-induced manner. Our results may be compatible with data indicating that D4-GDI is a protein that participates in T cell signaling cascades.

Additionally, we demonstrated that mutated D4-GDI increased the proliferation capacity of T cells induced in the blastic phase by Con A. This result is interesting because the mutation was originally identified in a leukemic cell line. The findings of the capping and CFSE assays demonstrate that this mutation may affect invasion, migration and proliferation, thereby increasing the malignancy of the leukemic cells. Li et al. (42) have reported that RhoH encoded by a hematopoiesis-specific Rho-related gene was identified in a fusion transcript with bcl6 in lymphoma cell lines and that reduction of RhoH levels in T cells augmented the response to Rac activation. Furthermore, RhoH is down-regulated after PMA treatment in T,1 cells after activation by anti-CD3 (42). The relationship between D4-GDI and RhoH is unknown, but some possible functions of the two molecules in the localization and response to TCR stimulation in T cells are suggested.

These findings suggest that D4-GDI may have important functions, such as the induction of T cell migration, adhesion and/or proliferation in the inflammatory foci, in cellular immune responses to cytopathic pathogens. Further studies are needed to investigate the molecular mechanism responsible for the effects of wild-type and mutated D4-GDI over-expression on cellular immune systems. This line of research could lead to a clearer understanding of the role of D4-GDI expression in hematopoietic cells.

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Abbreviations

ATCC American Type Culture Collection
BCG bacille Calmette-Guerin
CFSE carboxyfluorescein diacetate succinimidyl ester
CFU colony-forming units
ERG ezrin/radixin/moesin
GAP GTPase-activating protein
GDI guanosine diphosphate-dissociation inhibitor
GDP guanosine diphosphate
GDS GDP dissociation stimulator
GST glutathione

References


