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RhoH/TTF Negatively Regulates Leukotriene Production in Neutrophils

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Leukotriene B4 (LTB4) is an important proinflammatory lipid mediator generated by neutrophils upon activation. GM-CSF stimulation is known to enhance agonist-mediated LTB4 production of neutrophils within minutes, a process called “priming”. In this study, we demonstrate that GM-CSF also limits the production of LTB4 by neutrophils via a transcriptional mechanism at late time points. We identified hemopoietic-specific Ras homologous (RhoH)/translocation three four (TTF), which was induced following GM-CSF stimulation in neutrophils, as a key regulator in this process. Neutrophils derived from RhoH/TTF-deficient (RhoH−/−) mice demonstrated increased LTB4 production upon activation compared with normal mouse neutrophils. Moreover, neutrophils from cystic fibrosis patients expressed enhanced levels of RhoH/TTF and generated less LTB4 upon activation compared with normal human neutrophils. Taken together, these data suggest that RhoH/TTF represents an inducible feedback inhibitor in neutrophils that is involved in the limitation of innate immune responses.

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2 Deceased. The remaining authors dedicate this work to the memory of Dr. Andrew Ziemiecki.
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Materials and Methods

Reagents

Human GM-CSF was purchased from Novartis Pharma. Mouse GM-CSF as well as human IL-3, IL-8, and IFN-γ were from R&D Systems Europe and human complement factor C5a from MBL International. Mouse C5a was from Hycult Biotechnology and mouse IL-3 from BD Biosciences. LTB4 and the signaling inhibitors Gö6976 (PKC inhibitor), PD98059 (MEK inhibitor), SB 203580 (p38 MAPK inhibitor), and LY294002 (PI3K inhibitor) were obtained from Calbiochem. Polyclonal anti-RhoGDI-2 Ab was from Cell Signaling Technology. Polyclonal anti-Rac2 and anti-RhoA Abs as well as anti-CD16 mAb were from Santa Cruz Biotechnology. Anti-GAPDH mAb was from Chemicon International. HRP-conjugated secondary Abs were from Amersham Biosciences. FITC and tetramethylrhodamine isothiocyanate-conjugated anti-mouse and anti-rabbit secondary Abs were purchased from Molecular Probes (Invitrogen). Anti-Gr-1 mAb was from Miltenyi Biotec. Cycloheximide (CHX) and all other reagents were from Sigma-Aldrich.

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Mice
Rhoh−/− mice were generated and provided by Dr. C. Brackebusch (Department of Molecular Pathology, University of Copenhagen, Copenhagen, Denmark) (13). For all experiments, 6- to 8-wk-old mice with a C57BL/6J background were used. Mice were maintained under pathogen-free conditions. All animal experiments were reviewed and approved by the Animal Experimentation Review Board of the State of Bern.

Cells
Mature human blood neutrophils were isolated from peripheral blood of healthy donors and cystic fibrosis patients by Ficoll-Hypaque centrifugation (14–17). Briefly, PBMC were separated by centrifugation on Ficoll-Hypaque (Seromed-Fakola). The lower phase, mainly granulocytes and erythrocytes, was treated with erythrocyte lysis solution (155 mM NH₄Cl, 10 mM Hepes, and 0.1 mM EDTA (pH 7.5). The resulting cell populations contained >95% mature neutrophils as assessed by staining with Diff-Quik (Medion) and light microscopy analysis.

Mouse mature neutrophils were also isolated from wild-type and Rhoh−/− mice. Neutrophils were positively selected from bone marrow (obtained from femur and tibia of the hind legs) using anti-Gr-1 mAb as described previously (18). The purity of the resulting mouse neutrophil populations was >95%.

Cell cultures
Human and mouse neutrophils were cultured at 1 × 10⁶/ml in complete culture medium (RPMI 1640 containing 10% FCS) and, where indicated, treated with GM-CSF (50 ng/ml), IL-3 (50 ng/ml), IFN-γ (250 U/ml), and C5a (100 nM) for the indicated time periods. The signaling inhibitors Gö6976 (500 nM), PD98059 (50 μM), SB203580 (30 μM), and LY294002 (10 μM) were added 30 min before cytokine stimulation of neutrophils. CHX was used at 50 μg/ml. For LTB₄ production, neutrophils were primed with GM-CSF or IL-3 for 30 min and subsequently stimulated with C5a for 30 min.

LTB₄ immunoassay
LTB₄ concentrations were measured in human and mouse neutrophil supernatants by using commercial ELISA kits (Assay design; LuBioScience) according to the manufacturer’s recommendations.

Gene expression profiling
The transcriptional repertoire of mature human blood neutrophils cultured for 7 h in the presence and absence of GM-CSF was analyzed using HG-U95Av2 GeneChips (Affymetrix) as described previously (19). Each array was performed in triplicate.

RT-PCR
Neutrophils (1 × 10⁶) were washed with PBS and total cellular RNA was isolated using TRizol solution (Invitrogen). Approximately 1 μg of total RNA was reverse-transcribed using oligo-(dT)₁₂ priming (Promega) and Superscript Reverse Transcriptase (Invitrogen). Primers for human RhoH/TTF (5’-CCG CCG ATG CAT GTG TAC GTC GGT G-3’ and 5’-GCC CGG ATC CAT GGT GAG GGT G-3’) amplifications were synthesized (MWG Biotech). The cycling parameters for RhoH/TTF cDNA amplification were as follows: 95°C for 30 s, 60°C for 1 min, and 72°C for 30 s; and 72°C for 7 min. Primers for GAPDH were described previously (20). RhoH/TTF (580 bp) and GAPDH (417 bp) PCR products were separated on 1% agarose gels and visualized by means of ethidium bromide staining. The identity of the RhoH/TTF PCR product was confirmed by sequence analysis.

Quantitative real-time PCR (TaqMan)
One microgram of total RNA from mouse neutrophils isolated with SV Total RNA Isolation System (Promega) was used for RT-PCR (iPmProm-H Reverse Transcriptase System; Promega) using random primer for amplification. After cDNA synthesis, 25 ng of cDNA were used for additional analysis. Primers were designed based on the published sequence of mouse Rhoh (National Center for Biotechnology Information (NCBI), accession no. NM_0018081105; Gene ID: 74734) and synthesized by Microsynth Laboratory. Sequences were as follows: 5’-CTC AAT CAA GTG GTG GCG CGC TGT GTT GAC TGG TCT GTC GCC GCT C-3’ and 5’-GGA ATT CTT AGT AGA AGA AGA AGA AGA AGA AGA AGA CTC ACT C-3’. The reporter dye was SYBR green. The PCR buffer was from Bio-Rad. The run was performed on the iQ5 Multicolor Real-Time PCR detection system from Bio-Rad. The cycling conditions were as follows: 95°C for 15 min (1 cycle), 95°C for 15 s and 58°C for 1 min (40 cycles). The Bio-Rad iQ5 2.0 Standard edition optical system software was used to analyze real-time and endpoint fluorescence. The fold induction values were obtained according to the ΔΔCt method.

Production of rRhoH/TTF and polyclonal anti-human RhoH/TTF Ab
The RhoH/TTF cDNA was obtained by RT-PCR using cDNA from Jurkat cells. Primers were designed based on the published sequence (NCBI, accession no. NM_004310; Gene ID: 399) and synthesized by MWG Biotech. Sequences were as follows: 5’-CCG CCG ATG CAT GTG TAC GTC GGT G-3’ and 5’-GCC CGG ATC CAT GGT GAG GGT G-3’. The resulting cell populations contained >95% mature neutrophils as assessed by staining with Diff-Quik (Medion) and light microscopy analysis.

Immunoblotting
Gel electrophoresis and immunoblotting were performed as described previously (18, 22, 23). Cells were lysed with 2× loading buffer (Invitrogen) and sonicated. After electrophoresis of the separated proteins, the filters were incubated overnight with anti-RhoH/TTF (1/5000), anti-Rac2 (1/1000), anti-RhoA (1/1000), and anti-RhoGDI-2 (1/1000) Abs at 4°C in TBS/0.1% Tween 20/5% nonfat dry milk. For loading controls, stripped filters were incubated with anti-GAPDH (1/5000) mAb. Filters were developed by an ECL technique (ECL Kit; Amersham Biosciences) according to the manufacturer’s recommendations.

Confocal laser scanning microscopy
Indirect immunofluorescent stainings were conducted on 5-μm-thick paraformaldehyde-fixed, paraffin-embedded tissue sections from appendixitis and ulcerative colitis patients (18, 24). Slides were dried at 52°C for 2 h and deparaffinized using Neoclear Solution (Merck), ethanol (100, 90, 80, and 60, and 40%), and distilled water at room temperature. Following microwave treatment in buffer solution (10 mM sodium citrate (pH 6.0)), slides were washed in distilled water. To prevent nonspecific binding, slides were incubated in blocking solution (33% human IgG polyvalent, 33% normal goat serum, 33% BSA (7.5% in PBS) and 1% human IgG whole molecule) at room temperature for 1 h. Immunostainings with primary Abs were performed at 4°C overnight. RhoH/TTF was stained by using affinity-purified anti-RhoH/TTF Ab (colitis: 1/20; appendixitis: 1/25). To specifically detect neutrophils in these tissues, we used mouse anti-CD16 mAb (colitis: 1/20; appendixitis: 1/25). Following incubation with primary Abs, tissues were incubated with appropriate FITC- and tetramethylrhodamine isothiocyanate-conjugated secondary Abs (1/400) in the dark at room temperature for 1 h. The anti-fading agent Dako Mounting Medium (DakoCytomation) was added. Slides were covered by coverslips and analyzed by confocal laser scanning microscopy (LSM 510; Carl Zeiss) equipped with argon and helium-neon lasers.

Statistical analysis
ANOVA followed by Tukey’s HSD test was used to compare mean levels. A p value of <0.05 was considered statistically significant. Mean levels are presented together with SEM.

Results
GM-CSF rapidly induces RhoH/TTF expression in mature neutrophils
To gain an understanding of the molecular processes that occur during infection in neutrophils, we screened 12,599 genes in these cells for changes in gene expression following 7-h GM-CSF stimulation (19). There were four genes, which were up-regulated ≥20-fold: SOCS-1, RhoH/TTF, CD69, and CD44 (Fig. 1A). RhoH/TTF mRNA was induced 23.43-fold as assessed by the used microarray assay. The full microarray data set is provided at the

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GM-CSF rapidly induces RhoH/TTF in mature neutrophils. A, Relative expression of genes induced upon 7-h GM-CSF stimulation as assessed by microarray analysis. B, Time-dependent RhoH/TTF mRNA expression upon GM-CSF stimulation as assessed by RT-PCR. C, RhoH/TTF protein expression upon 8-h stimulation with different cytokines as assessed by immunoblotting. D, Time-dependent RhoH/TTF protein expression upon GM-CSF stimulation as assessed by immunoblotting. In contrast to RhoH/TTF, levels of RhoGDI-2 were not regulated by GM-CSF. E, RhoH/TTF, RhoA, and Rac2 protein expression upon 6-h GM-CSF stimulation as assessed by immunoblotting. F, Pharmacological inhibition of PKC, PI3K, and p38 MAPK blocks GM-CSF-induced RhoH/TTF protein expression as assessed by immunoblotting. Mean levels of three independent experiments (A) and representative examples of at least three independent experiments in (B–F) are shown.

To test whether activation of protein kinase C (PKC), PI3K, and/or MAPK pathways are involved in GM-CSF-mediated RhoH/TTF expression in neutrophils, neutrophils were preincubated with defined kinase inhibitors. Gö6976, an inhibitor of PKC, SB203580, a selective inhibitor of p38 MAPK, and LY294002, an inhibitor of PI3K, blocked RhoH/TTF expression (Fig. 1F), suggesting that PKC, p38 MAPK, and PI3K pathways are involved in the transcriptional activation of the gene. In contrast, PD98059, an inhibitor of MEK proximal to p42/44 MAPKs had no blocking effect.

**Neutrophils express RhoH/TTF under inflammatory conditions**

Because RhoH/TTF is markedly up-regulated under conditions of GM-CSF exposure, we hypothesized that neutrophils under inflammatory conditions should express higher levels of RhoH/TTF. Indeed, compared with normal neutrophils, blood neutrophils from patients with cystic fibrosis had increased RhoH/TTF protein levels (Fig. 2A). Moreover, to demonstrate RhoH/TTF expression in neutrophils under in vivo inflammatory conditions, we analyzed neutrophils in tissue sections of patients suffering from appendicitis and ulcerative colitis, respectively. Neutrophils were identified using anti-CD16 mAb. Neutrophils expressed RhoH/TTF in these tissues, and it appeared that RhoH/TTF was located both in the cytosol and at the cell membrane (Fig. 2B). In the ulcerative colitis sections, we also obtained evidence for strong RhoH/TTF expression in infiltrating CD16-negative cells, most likely eosinophils.

**LTB₄ is decreased in neutrophils from cystic fibrosis patients and increased in mouse neutrophils defective in RhoH**

Human neutrophils are known to generate LTB₄ upon priming with GM-CSF and subsequent short-term stimulation with C5a (2). Indeed, significant LTB₄ production was seen in GM-CSF-primed cells only, whereas single stimulation with neither GM-CSF nor C5a resulted in a functional response (Fig. 3, left panel). Interestingly, neutrophils from patients suffering from cystic fibrosis were defective in LTB₄ production upon GM-CSF priming and subsequent C5a stimulation (Fig. 3, right panel).
To obtain first evidence whether such a defective LTB₄ production could be due to increased RhoH/TTF expression, we performed a time-course experiment using normal neutrophils in the presence and absence of a protein synthesis inhibitor (CHX). C5a alone did not significantly induce LTB₄ production of freshly isolated neutrophils. However, pretreatment with GM-CSF for 30 min and subsequent C5a stimulation resulted in the generation of significant amounts of LTB₄ (Fig. 4A). CHX had no effect at this time point. In the absence of CHX, we observed that this priming effect was less after 1-h GM-CSF stimulation and was practically lost at the 2-h time point (Fig. 4A). In contrast, in the presence of CHX, neutrophils kept the capacity to generate LTB₄ after C5a stimulation (Fig. 4A). In these experiments, we monitored RhoH/TTF expression at all time points. As expected, RhoH/TTF was rapidly detectable (within 1 h) after GM-CSF stimulation, and CHX prevented GM-CSF-induced RhoH/TTF protein expression in neutrophils (Fig. 4A). Interestingly, reduced amounts of LTB₄ production were associated with increased RhoH/TTF expression at all time points up to 5 h after GM-CSF stimulation (Fig. 4B). Taken together, these data pointed to the possibility that RhoH/TTF could indeed play a limiting role in LTB₄ production of neutrophils.

The precise investigation of signaling mechanisms in neutrophils has been difficult because the cells are fragile and undergo rapid spontaneous apoptosis. Plasmid transfection appeared to be impossible in neutrophils (26), limiting the common approaches to study signaling pathways in these cells. Therefore, to investigate a possible function of RhoH/TTF in neutrophil’s leukotriene production, we assessed the capacity to generate LTB₄ in neutrophils derived from wild-type and Rhoh/H₁¹⁰₀₂/H₁¹⁰₀₂ mice. Similar to human neutrophils, wild-type mouse neutrophils rapidly induced the Rhoh gene following GM-CSF stimulation (Fig. 5A). Neutrophils from Rhoh/H₁¹⁰₀₂/H₁¹⁰₀₂ mice demonstrated no detectable Rhoh mRNA expression in these experiments (data not shown). Freshly isolated neutrophils from wild-type mice generated similar amounts of LTB₄ after GM-CSF priming and subsequent C5a stimulation (mean level: ~15,000 pg/ml) as seen using human neutrophils. Moreover, there was no significant difference between neutrophils derived from wild-type and Rhoh⁻/⁻ mice (Fig. 5B), consistent with the
observation that resting human neutrophils express no or only very little RhoH/TTF.

Because both human and mouse neutrophils consistently expressed more RhoH/TTF after GM-CSF stimulation, we also performed LTB4 release experiments in mouse neutrophils treated with GM-CSF for several hours. Because the priming effect of GM-CSF on human neutrophils did not last longer than 1 h (Fig. 4), neutrophils were primed with IL-3 and subsequently stimulated with C5a. Because maximal levels of Rhoh mRNA were seen after 3-h GM-CSF stimulation (Fig. 5A), we performed these experiments in 3-, 5-, and 8-h pretreated neutrophils. Strikingly, under these conditions of GM-CSF pretreatment and IL-3 priming, neutrophils from Rhoh-/- mice generated significantly more LTB4 after C5a stimulation compared with neutrophils from wild-type mice (Fig. 5C).

**Discussion**

Ras-like proteins constitute a protein superfamily that can be subdivided into five main families, one of these is the family of Rho proteins (27). The Rho family members are defined by the presence of a Rho-specific insert located between the G4 and the G5 boxes and involved in mediating protein-protein interactions (28). Rho proteins have been shown to participate in multiple signaling pathways (29). Most functional data have been reported for Rac1/2, RhoA, and cell division cycle 42 (Cdc42). In contrast, little is known about the function of the many other members of the Rho family, such as RhoH/TTF. Recently published work suggests that RhoH/TTF plays a key role in TCR signaling (13, 30). For instance, it has been demonstrated that Rhoh/TTF is required for recruitment of ZAP70 to the TCR. Consequently, RhoH/TTF deficiency resulted in decreased activation of phospholipase Cγ1 and impaired calcium influx (13), as well as in T cell proliferation defect (30). These data suggest that RhoH represents a positive regulator of TCR signaling.

In contrast to these findings obtained in T cells, RhoH/TTF has been described as a negative regulator of proliferation and engraftment of hemopoietic progenitor cells, perhaps due to the induction of apoptosis in these cells (10). We did not observe differences in the kinetics of neutrophil apoptosis between wild-type and Rhoh-/- mice (data not shown). When acting as a negative regulator, RhoH/TTF was suggested to antagonize Rac1/2-mediated signaling pathways (9, 10, 31). This assumption was supported by a study performed in Jurkat cells, in which retroviral RhoH/TTF gene transfer resulted in inhibition of Rac1 signaling (32). Clearly, besides differences in the cell types used and signaling pathways investigated, it cannot be excluded that differences in the methodological approaches account, at least partially, for the different conclusions that have been made regarding RhoH/TTF functions in the different studies.

We report in this study that RhoH/TTF is a negative regulator of LTB4 production in neutrophils. In these cells, RhoH/TTF is transcriptionally regulated by GM-CSF, which plays a key role in antibacterial defense mechanisms. We also show that RhoH/TTF is expressed in neutrophils under inflammatory conditions, such as cystic fibrosis, ulcerative colitis, and appendicitis. Rho family members have previously been reported to exert important functions in neutrophils. For instance, Cdc42 is translocated to lipid rafts, where it activates p38 MAPK upon LPS-induced activation in these cells (33). Similarly, we obtained evidence that a proportion of RhoH/TTF is located at the cell membrane of neutrophils under inflammatory conditions (Fig. 2B), pointing to the possibility that it translocates from the cytosol to the cell membrane upon activation. Indeed, RhoH/TTF has a conserved polybasic domain and a CAAX prenylation site (9, 34), which allows membrane targeting (35). How RhoH/TTF exerts its regulatory role in neutrophils remains to be investigated.

Rac2, another member of the Rho family, was found to be essential for primary (azurophilic), but not secondary and tertiary granule release of neutrophils (36). In addition, Rac2 was found to be crucial for superoxide production and chemotaxis in neutrophils (34). These data are in agreement with the increased susceptibility toward bacterial and fungal infections in functional Rac2-deficient mice and humans (37–39). Moreover, RhoA has been studied in neutrophils and is believed to be involved in integrin functions of neutrophils (40). RhoA may also induce stress fibers formation, which affects the efficacy of signal transduction via surface receptors (41). In a recent study, it was suggested that RhoA interacts with Cdc42, exerting both positive and negative signaling effects...
depending on the activation stage of neutrophils (42). Taken together, Rho family members are expressed in neutrophils and are crucial for several functions of these cells. They interact with each other and other signaling molecules; however, the molecular interactions between Rho family members and their effectors are largely not understood.

In summary, this study demonstrates elevated levels of RhoH/TF in neutrophils in association with GM-CSF exposure in vitro and in vivo. Therefore, GM-CSF does not only exhibit well-known proinflammatory activities, it also induces the expression of genes that limit inflammatory responses, such as suppressor of cytokine signaling 1 (see Fig. 1A) and RhoH/TF. RhoH/TF negatively regulates agonist-mediated LTβR production. Further studies are needed to understand the molecular mechanisms of transcriptional activation of the RhoH/TF gene. Moreover, the intracellular movement of RhoH/TF and its binding partners as well as the exact signaling mechanisms and possible additional cellular functions that are regulated by RhoH/TF in neutrophils remain to be investigated.

Disclosures
The authors have no financial conflict of interest.

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