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Proprotein Convertase FURIN Constrains Th2 Differentiation and Is Critical for Host Resistance against Toxoplasma gondii

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The proprotein convertase subtilisin/kexin enzymes proteolytically convert immature proproteins into bioactive molecules, and thereby they serve as key regulators of cellular homeostasis. The archetype proprotein convertase subtilisin/kexin, FURIN, is a direct target gene of the IL-12/STAT4 pathway and it is upregulated in Th1 cells. We have previously demonstrated that FURIN expression in T cells critically regulates the maintenance of peripheral immune tolerance and the functional maturation of pro-TGF-β1 in vivo, but FURIN’s role in cell-mediated immunity and Th polarization has remained elusive. In this article, we show that T cell–expressed FURIN is essential for host resistance against a prototypic Th1 pathogen, Toxoplasma gondii, and for the generation of pathogen-specific Th1 lymphocytes, including Th1–IL-10 cells. FURIN-deficient Th cells instead show elevated expression of IL-4R subunit α on cell surface, sensitized IL-4/STAT6 signaling, and a propensity to polarize toward the Th2 phenotype. By exploring FURIN-interacting proteins in Jurkat T cells with Strep-Tag purification and mass spectrometry, we further identify an association with a cytoskeleton modifying Ras-related C3 botulinum toxin substrate/dedicator of cytokinesis 2 protein complex and unravel that FURIN promotes F-actin polymerization, which has previously been shown to downregulate IL-4R subunit α cell surface expression and promote Th1 responses. In conclusion, our results demonstrate that in addition to peripheral immune tolerance, T cell–expressed FURIN is also a central regulator of cell-mediated immunity and Th1/2 cell balance.


Upon encountering the cognate Ag, naive CD4+ Th cells activate and polarize into functionally distinct subsets that include Th1, Th2, Th17, and T regulatory cells (Tregs). The fate of a naive Th cell depends on the length and strength of the Ag stimulus, as well as on surrounding cytokine milieu, both of which activate cellular signaling pathways and expression of Th subtype-specific genes (1). The first characterized subtypes, Th1 and Th2, determine the balance between cell-mediated and humoral immune responses. Th1 polarization is characterized by a strong Ag stimulus in the presence of the cytokine IL-12. These trigger the activation of transcription factor STAT4 and elevated T-box 21 (T-bet) expression, followed by secretion of the Th1 type effector cytokines. Th2 polarization, in contrast, is initiated by a weak Ag stimulus and IL-2/STAT5 signaling, resulting in up-regulation of GATA binding protein 3 (GATA-3) and IL-4–induced STAT6 activation (2). Th1 cells secrete IFN-γ and TNF cytokines that promote, for example, the activation of macrophages in intracellular infections, whereas the Th2 cytokines IL-4, IL-5, and IL-13 induce IgE class switching in B cells, as well as the activation of eosinophils, both of which are required to restrain parasitic infections (3).

Dysregulation of Th cells is associated with immune-mediated diseases, including infections, immunodeficiencies, autoimmunity, and allergic responses (4). Insufficient Th1 polarization, for example, increases susceptibility to mycobacterial infections, whereas activated Th1 cells are often overrepresented in autoimmune diseases (2). Consequently, much research effort has been aimed at identifying the detailed molecular mechanisms that control Th polarization events over the past two decades. We and others have previously shown that...
a proprotein convertase subtilisin/kexin (PCSK) family protease FURIN is a direct target gene for IL-12/STAT4 and TGF-β1, and it is highly expressed in human Th1 cells (5–7). PCSK enzymes are a family of nine proteases (PCSK-1, FURIN, PCSK4–7, MBTPSI, PCSK9) that cleave and convert their immature target proteins into biologically active forms by catalyzing endoproteolytic cleavage at a target site typically made up of basic amino acids arginine and lysine (8). Accordingly, PCSK enzymes play a key regulatory role in a multitude of biological events, including development and hormone function (9). Dysregulated proprotein convertase activity also contributes to pathogenic cell behavior; therefore, interfering with PCSK activity is currently being considered as a potential treatment for many diseases including atherosclerosis (10), rheumatoid arthritis (11), and multiple sclerosis (12).

PCSK enzymes are also central regulators in host defense; inhibiting PCSK activity can prevent the proteolytic activation of bacterial toxins and viral entry (8), and recently a FURIN-like convertase was shown to regulate human TLR7 processing and subsequent antiviral immunity (13). In addition, deleting PCSK1 expression in mice is associated with accelerated proinflammatory responses (14), whereas PCSK7 participates in rescuing the unstable MHC class I molecules on dendritic cells (DCs) (15) and regulates the bioavailability of TGF-β1a cytokine in zebrafish (16).

Biochemical analyses have also demonstrated that the PCSK controlled proprotein cascades are important in the functional maturation of several proteins critical for host defense such as integrins, matrix metalloproteinases, and cytokines (9, 17).

We have previously shown that the T cell–expressed proprotein convertase FURIN is essential for maintaining Treg-mediated peripheral immune tolerance. CD4cre-fur1mice develop an age-related systemic autoimmune disease that is characterized by circulating autoantibodies, overtly activated CD4+ and CD8+ T cells, and overproduction of both Th1 and Th2 cytokines, IFN-γ, IL-4, and IL-13 (18). The accelerated immune responses in CD4cre-fur1mice could be chiefly attributed to reduced bioavailability of a central anti-inflammatory cytokine TGF-β1 in T cells. Notably, the role of FURIN in controlling the Th cell subsets appears complex and is not limited to CD4FOXP3+Tregs; inhibiting FURIN by a recombinant inhibitor (α-1-antitrypsin Portland) or small interfering RNA can also abrogate the production of the Th1 effector cytokine IFN-γ by human CD4+ T cells (5).

In this study, we show that FURIN expression in T cells is critical for ensuring an appropriate immune response against a prototypic Th1 parasite, Toxoplasma gondii. Specifically, our data demonstrate that FURIN expression is needed for adequate Ag-specific Th1 cell generation, and lack of FURIN expression in T cells results in the inherent upregulation of IL-4R subunit α (IL-4Rα) on the cell surface and dominance of Th2 polarization. Using Strep-tag purification and mass spectrometry (MS), we further show that FURIN interacts with a Th1-promoting and IL-4Rα–inhibiting Ras-related C3 botulinum toxin substrate (RAC)/dedicator of cytokinesis 2 (DOCK2) protein complex and regulates T cell cytoskeleton dynamics by increasing its activity.

**Materials and Methods**

**Mice**

T cell–specific FURIN conditional knockout (cko; CD4cre-fur1mice on C57BL/6 background have been described earlier (18, 19); IL-12p40 KO animals were purchased from Taconic. For OVA-specific Th polarization experiment, CD4cre-fur17/OTII mice, which have OVA-specific CD4+ T cells. All mice were housed under pathogen-free conditions in accordance with the National Institutes of Health Animal Care and Use Committee (National Institutes of Health, Bethesda, MD) or the National Animal Experiment Board (Finland).

**Cell culture and transfections**

Jurkat E6-1 T cells (ATCC TIB-152) were cultured in RPMI 1640 medium and HeLa cells (ATCC CCL2) in DMEM medium, both supplemented with 10% FBS, l-glutamine, and antibiotics. To generate the stable wild type (wt) and inactive FURIN (mutant [mut])-expressing cell lines, we electroporated Jurkat E6-1 T cells (1025transfectants per T cell) with DNA constructs pcDNA3.1-hFURIN-D153Amut-StrepIII, or pcDNA3.1-StrepIII as control (a kind gift from Prof. Jukka Westermarck, Turku Centre for Biotechnology). Geneatin antibiotic (600 μg/ml) was added to the cells cultures 3 d posttransfection. cDNA-expressing cell lines were then selected with clonal dilution, and constant expression of recombinant protein was evaluated by Western blot analysis (anti-FURIN MON-152; Enzo LifeSciences). Cells lines with wt and mut FURIN expression were subjected to functional studies and F-actin polymerization studies. HeLa cells were transiently transfected with 0.5–1 μg pcDNA-RAC1-V5, pcTI-DOCK2-FLAG (kind gifts from Prof. Yoshinori Fukui, Kyushu University, Fukuoka, Japan), and pcDNA3.1-h-FURIN-MYC-His using FuGENE 6 Transfection Reagent (Promega).

**Cell purification, cytokine measurements, and flow cytometry**

To study OVA-induced Th1/Th2 polarization, we purified naïve CD4+ CD44+CD62L+ T cells using flow cytometry from CD4cre-fur1 or littermate control mice on an OTII background. Cells were labeled with CFSE (Invitrogen) and stimulated with graded concentrations of OVA- peptide ([5–1000 nM) and sorted splenic CD11c+CD49b+ DCs under neutral conditions. After 3.5 d, the cells were activated with PMA (10 ng/ml) and ionomycin (1 μM; EMD), and intracellular expression of IFN-γ, IL-4, and IFN-γ, IL-4, and CFSE was analyzed with flow cytometry.

To provide a phenotypic T cell activation studies, naïve T cells were isolated with CD4+ CD62L− T Cell Isolation Kit (Miltenyi) from spleen and lymph nodes. Cells were activated for 72 h with plate-bound anti-CD3 and -CD28 (10–10 μg/ml) Abs in serum-free X-VIVO 20 medium (Lonza). IL-12 (10 ng/ml), TGF-β1 (0.5 ng/ml), or anti-IL-4 (2 μg/ml) were added to the cells cultures during the activation. Cytokines from cell culture medium were measured with cytometric bead array Th1/2/17 kit (BD Biosciences) or IL-4 ELISA (Peprotech), and R&D systems collected quantities for the real-time PCR (Bio-Rad). For the detection of pERK, CD4+ T Isolation Kit (L3T4; Miltenyi) were used to isolate CD4+ T cells from spleen and lymph nodes. Cells were stimulated with soluble anti-CD3 (10 μg/ml for 5–60 min, and phosphorylation of ERK was detected by Western blot using ERK and pERK primary Abs (Cell Signaling Technology) and anti-rabbit HRP-biotin conjugated secondary Ab (R&D Systems). Signal intensities were analyzed using National Institutes of Health ImageJ software. IL-4Rα cell-surface expression was analyzed from spleens and lymph nodes by staining the cells with anti–IL-4Rα (BD Biosciences), -CD4, and -CD62L (eBioscience) Abs, and analyzed by flow cytometry. To detect tyrosine phospho-STAT6, we purified naïve CD4+ T cells from spleen and lymph nodes with the CD4+CD62L− T cell isolation kit (Miltenyi) and activated them with IL-4 (1 or 10 ng/ml) for 15 min. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.5%–10% methanol overnight. Staining was performed in 0.5% Triton X-100, anti-BSA/PBS with anti-p-STAT6 Ab (BD Biosciences). All FACS analyses were performed using FACS Calibur or FACS Canto instrument (BD Biosciences), and data were analyzed with FlowJo software (Tree Star).

**Quantitative real-time PCR**

Total RNA was isolated with RNeasy kit (Qiagen) and reverse transcribed into cDNA with iScript kit (BioRad). Gene expressions were analyzed using Bio-Rad EvaGreen supermix and CFX96 instrument (BioRad). Primer sequences and size of amplicons: T-bet (110 bp); forward, 5'-TCAAC-CAGCACAGCAGAG-3', reverse, 5'-AAAAATCTCTGTTAAGTGGCTT-GTG-3'; Gata-3 (75 bp): forward, 5'-TTATAAGCCCAAGGCAAG-3', reverse, 5'-TGGTTGCTGGTGCTGACAGTTC-3'; Il4ra (102 bp): forward, 5'-GACTGCTAGTGGAGGACATA-3', reverse, 5'-CAGTCCACAGGAGCATC-3'; Il4ra (143 bp): forward, 5'-GATGATCCCTGGAGGACATA-3', reverse, 5'-TCAGTTCTCTGTTCCATTCA-3'. The expression of each gene was normalized to ribosomal 18s gene. For FURIN and B2M housekeeping gene expression analysis in splenocytes after T ge ndili infection, predesigned primers and probes from Applied Biosystems were used.

**Immunofluorescence**

Three days posttransfection, HeLa cells were fixed on coverslips with parafomaldehyde and stained with rabbit anti-FURIN (a kind gift from Prof. John Creemers, Katholieke Universiteit Leuven, Leuven, Belgium) and mouse anti-IL-4 for IAC (Invitrogen). Nucleus was stained with DAPI, and specific protein expression was visualized with anti-rabbit Texas Red or anti-mouse Alexa 488 (both Life Technologies), using ApoTome microscope and AxioVision software (Zeiss).

![Image](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/)

**The Journal of Immunology** 5471

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Immunoprecipitation and Western blotting

Transfected HeLa cells were lysed (lysis buffer: 50 mM Tris pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 mM NaF, 1 mM TCEP, and Complete Mini protease inhibitor from Roche) and preclariye with protein G-Sepharose 4 FastFlow beads (GE Healthcare). Anti-FLAG Ab was used to capture DOCK2 and anti-MYC for FURIN, in parallel with Ab isotype and resin controls (all from Sigma-Aldrich). Protein eluates were separated with SDS-PAGE gel and transferred to nitrocellulose membrane. Immunodetection was performed using anti-FLAG or anti-MYC primary Abs and anti-mouse HRP-conjugated secondary Ab (R&D Systems). Visualization was done using the ECL Western blotting detection kit (GE Healthcare) and AGFA CP1000 imaging system.

F-actin polymerization

FURIN wt and control Jurkat E6-1 T cell lines were starved in RPMI 1640 supplemented with 1% FBS and stimulated with 250 ng/ml stromal cell-derived factor-1α (SDF-1α; PeproTech) for 0–120 s. Cells were immediately fixed with 4% paraformaldehyde and permeablized with 0.1% Triton X-100, stained for polymerized F-actin (Phalloidin-FITC; Sigma-Aldrich), and analyzed with flow cytometry.

In vivo challenge with T. gondii

CD4cre-furf, IL-12p40 KO, and littermate control mice (6–10 wk old) were challenged i.p. with 2 × 10⁶ pfu of the avirulent ME49 strain of T. gondii. Nine days postinfection, the FURIN expression was quantified in splenic CD4⁺ T cells by Q-RT-PCR, and serum cytokines were measured with CBA (BD Biosciences). Survival of FURIN T cell KO and littermate control animals was monitored for 100 d and quantified using log-rank (Mantel–Cox) test. Some of the infected animals were sacrificed on 38 d postinfection to analyze the number of T. gondii cysts in brain, as well as Ag-specific Th1/Th1–IL-10 polarization and cytokine production. In brief, brains were isolated and homogenized by sequential passage through 19- and 21-gauge needles, and the number of cysts was determined microscopically. Ag-dependent cytokine production was induced ex vivo by stimulating splenocytes in complete RPMI 1640 + 10% FBS with soluble T. gondii Ag (STAg; 5 μg/ml) for 72 h. For flow cytometry, cells were stained for CD4, IFN-γ, and IL-10 (BD Biosciences). Cytokines were measured from cell culture supernatants with CBA (BD Biosciences).

Sample preparation for MS

Cell membrane fractions from Jurkat T cell lines were isolated (Mem-PER Eukaryotic Membrane Protein Extraction Kit; Thermoflu Scientific) and FURIN, as well as associated proteins, were affinity purified from lysates with immobilized Ag against cysteine protease (IBA). The presence and purity of recombinant FURIN in elutes was verified with both anti-FURIN (MON-152; Enzo Life Sciences) and anti-Strep (IBA) Abs (data not shown). Eluted proteins were separated by one-dimensional SDS-PAGE gel (Miniprotein precast gel; Bio-Rad) and visualized by silver staining.

Mass spectrometry

Target bands were cut from silver-stained gels, and after enzymatic protein digestion and extraction, peptides were identified by MS (Proteomics Facility, Turku Centre for Biotechnology). Analysis was performed by liquid chromatography/electrospray ionization–tandem MS on a nanoflow HPLC system coupled online to an Orbitrap Velos MS instrument. Database searches were performed by Mascot (version 2.2.6) against SwissProt (UniProt) protein sequence database (version 2010_09). Scaffold 3 software (Proteome Software) was used to further analyze identified proteins. Data were filtered through validation parameters (i.e., m.w. match, minimum 2 unique peptides, minimum ~10% coverage).

Statistical analysis

Data represent mean ± SEM. Statistical significance was determined by nonparametric Mann–Whitney U test for mouse experiments and by two-tailed Student t test for cell line experiments, if not indicated otherwise. Survival after T. gondii infection was analyzed with log-rank (Mantel–Cox) test. The p values < 0.05 were considered statistically significant.

Results

T cell–expressed FURIN is essential for host resistance against T. gondii

We initially identified FURIN as a novel IL-12 target gene using microarray expression analysis on human peripheral blood T lymphocytes (5). Subsequent experiments showed that the FURIN mRNA and protein are particularly highly expressed in Th1 cells and that FURIN is directly regulated by STAT4 chromatin binding, rather than in a T-bet–dependent manner (20). T. gondii is an obligate intracellular parasite that triggers a profound Th1-mediated cellular immune response characterized by elevated production of IFN-γ and TNF cytokines. We first assessed whether T. gondii infection induces FURIN expression in splenic CD4⁺ T cells by infecting both wt and IL-12–deficient mice with avirulent T. gondii ME-49 strain parasites. In line with previous in vitro data showing an IL-12–dependent FURIN regulation (5), parasite-induced FURIN expression was significantly impaired in IL-12–deficient animals at 9 d postinfection (Fig. 1A).

To study the potential role of FURIN in this prototypic Th1–inducing infection, we next infected mice in which FURIN was deleted specifically in T cells (CD4cre-furf) with T. gondii. Presence or absence of FURIN in T cells did not significantly influence survival or serum cytokine levels in the IL-12–dependent (21, 22), acute phase of parasite infection (Fig. 1B and Supplemental Fig. 1). However, T. gondii–infected CD4cre-furf mice had modestly reduced numbers of IL-10–producing Th1 cells at 9 d postinfection (data not shown). Later in the course of infection, all infected CD4cre-furf animals became moribund and ultimately succumbed to the infection (Fig. 1B).

To better define the role of FURIN in mounting appropriate T. gondii–specific CD4⁺ T cell responses, we stimulated splenocytes from infected CD4cre-furf or littermate mice with STAg for 72 h (Fig. 1D). Total splenocyte cell number was reduced in the declining CD4cre-furf mice, but CD4⁺/splenocyte ratios in the spleen were similar to those of the littermate control animals. The resistance to T. gondii infection is critically dependent on an adequate Th1 response and IFN-γ/TNF cytokine production (24–26), but also on the generation of Th1–IL-10⁺ cells, which restrict the magnitude of the infection-induced inflammation (24, 27). STAg stimulation resulted in an increase of the numbers of both Th1 and Th1–IL-10 cells in control mice, whereas the generation of Ag-specific Th1 and Th1–IL-10 cells was significantly reduced in splenocytes isolated from CD4cre-furf mice, indicating that FURIN promotes cell-mediated immune responses (Fig. 1D, 1E).

Profiling the Ag-stimulated splenic cytokine production further showed a lower trend of IFN-γ levels accompanied with a significantly reduced production of another Th1 cytokine, TNF, but unchanged secretion of the innate immunity cytokines IL-6, IL-12, and MCP-1 (Supplemental Fig. 2). Together, our data demonstrate a critical role for proprotein convertase FURIN in T cell–mediated host resistance against T. gondii and in the generation of pathogen-specific Th1 and Th1–IL-10 responses.

FURIN regulates the Th1–Th2 balance

The failure of FURIN-deficient T cells to generate appropriate protective Th1 response prompted further investigation. To this end, we crossed CD4cre-furf mice with TCR transgenic (OTII) mice and stimulated the Ag-specific naïve CD4⁺ T cells (OTII⁺ CD4⁺CD62L⁺CD44⁺) with varying concentrations of cognate antigenic peptide (OVA), and analyzed the production of Th subtype cytokines and cell proliferation. As reported previously (28), in neutral culture conditions, low-dose Ag (5 nM) drove a predominant Th2 polarization of wt CD4⁺ cells, as attested by
high IL-4/IFN-γ ratio (Fig. 2). Conversely, increasing Ag (10–100 nM) favored generation of Th1 (IFN-γ+) cells. At a low OVA concentration, FURIN cKO CD4+ cells showed modestly enhanced cell proliferation and increased the IL-4+ Th2 cell proportions. Strikingly, stimulation of FURIN-deficient T cells to increasing Ag (10–100 nM) failed to induce normal proportions of IFN-γ+IL-4+ Th1 cells. In contrast, a marked overabundance of IL-4 production sustained upon increasing the Ag dose up to 100 nM. These data show that, in neutral culture conditions, FURIN-deficient T cells have an intrinsic impairment in Th1 and dominance of Th2 differentiation, which could be a result of altered sensitivity to the effects of Ag dose or dysregulated cytokine signaling.

Th2 polarization in FURIN-deficient CD4+ T cells is independent of TGF-β1 and is resistant to IL-12 inhibition

One aspect of the function of FURIN is its role in the maturation of pro–TGF-β1 through a site-specific proteolytic cleavage (29). Because TGF-β1 can have diverse effects on lymphocyte activation, we next addressed the possibility that the effect of FURIN was related to TGF-β1 or other serum cytokines (30). Naive wt and FURIN cKO T cells were activated with a strong polyclonal TCR stimulus (anti-CD3/28 10+10 μg/ml) in serum-free cell culture conditions, and IL-4 production was measured at different time points (Fig. 3A). Lack of FURIN expression was not associated with enhanced apoptosis, because cell numbers and Bcl-2 mRNA expression were unaffected (data not shown). Naive FURIN cKO CD4+ cells produced significantly greater amounts of IL-4 as early as 24 h after TCR activation, indicating that FURIN deficiency results in accelerated Th2 responses also upon a strong polyclonal TCR activation and independently of serum cytokines.

We next investigated whether FURIN-deficient T cells respond correctly to Th1- and Treg/Th17-inducing cytokines IL-12 and TGF-β1, respectively (Fig. 3B, 3C). In keeping with the increased levels of IL-4 as early as 24 h after TCR activation, indicating that FURIN deficiency results in accelerated Th2 responses also upon a strong polyclonal TCR activation and independently of serum cytokines.

The Journal of Immunology 5473

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

T cell–expressed FURIN is critical for the host defense to *T. gondii*. (A) wt and IL-12p40 KO (n = 6) mice were left uninfected (naive, n = 1) or infected (n = 5) i.p. with 20 *T. gondii* cysts (ME-49). Nine days postinfection, FURIN and B2M housekeeping gene expressions in splenic CD4+ T cells were determined with Q-RT-PCR. FURIN expression was normalized to B2M, and relative expression in uninfected wt CD4+ cells was arbitrarily set as 1. (B) Ten-week-old CD4cre-furf/f and littermate control mice were inoculated i.p. with 20 *T. gondii* ME-49 cysts. Survival was analyzed with log-rank (Mantel–Cox) test between FURIN cKO (n = 6) and littermate controls (n = 3); experiment was repeated twice with identical results. (C) Brain cysts were calculated from moribund FURIN cKO mice (n = 6) and littermate controls (n = 4) on day 38 postinfection. (D and E) Splenocytes from *T. gondii*-infected FURIN cKO (n = 6) and littermate (n = 4) mice were left nonstimulated (ns) or stimulated ex vivo with STAg (5 μg/ml) for 72 h. Production of IFN-γ and IL-10 was measured with flow cytometry in CD4+ T cells; representative cytokine stainings (D) and scatter plots (E) are shown. (A–E) Mean ± SEM. Statistics were calculated with Mann–Whitney *U* test except for survival analysis.
remained Th2 biased (elevated GATA-3 expression and IL-13 levels) also in the presence of exogenous TGF-β1, but responded by showing downregulation of IL-4. Altogether, we conclude that the FURIN-deficient T cells respond correctly to IL-12 and TGF-β1 cytokines, and the inherent Th2 bias is independent of serum and resistant to Th1 and Th17/Treg polarization-inducing cytokines. These findings suggest that the FURIN-deficient Th cells could hyperpolarize to Th2 phenotype as a result of sensitized responses to Th2-promoting signals, such as autocrine IL-4 production.

**FURIN inhibits the cell-surface expression of IL-4Ra in naive CD4+ cells**

Previous work has also shown that the strength of TCR signaling can affect Th1 versus Th2 commitment by influencing the degree of ERK activation; early Th2 polarization events are inhibited by an

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**FIGURE 2.** FURIN expression in CD4+ T cells inhibits Th2 polarization. Naive CD4+ T cells (CD4+CD44loCD62L-) from CD4cre-furf/fOTII or littermate control (furf/f-OTII) mice were labeled with CFSE and stimulated with graded concentrations of OVA-peptide/splenic DCs under neutral cell culture conditions. After 3.5 d, the primed T cells were stained for intracellular IFN-γ and IL-4. Cytokine production (A) and proliferation rate (B) were measured with flow cytometry. Experiment was performed twice with identical results.

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**FIGURE 3.** Th2 polarization in FURIN-deficient CD4+ T cells is independent of serum cytokines and resistant to IL-12 and TGF-β1. Naive CD4+CD62L+ T cells from CD4cre-furf/f (cKO) or littermate control (wt) mice were stimulated with plate-bound anti-CD3/28 (10+10 μg/ml) and cultured in serum-free medium in the presence or absence of IL-12 (10 ng/ml) or TGF-β1 (0.5 ng/ml) as indicated for 72 h. (A) IL-4 production was measured with ELISA at different time points (n = 2–3 for both groups). The p values were determined with Student t test, *p < 0.05, **p < 0.01. (B) T-bet and GATA-3 expressions were measured with Q-RT-PCR. (C) Cytokines from cell culture supernatants were measured with CBA Th1/2/17 kit (BD Biosciences). Shown are representative experiments of two identical performed (B and C). Error bars indicate SEM.
elevated ERK activation upon a strong Ag stimulus (28). To investigate mechanisms by which FURIN normally inhibits Th2 differentiation, we measured TCR-dependent ERK activation and found that ERK phosphorylation was enhanced, not impaired, in FURIN-deficient CD4+ cells (Fig. 4A). Moreover, FURIN-dependent Th2 bias was evident also in the presence of Th1 favoring strong TCR induction (Fig. 3). Thus, it seems unlikely that impairment of TCR signaling underlies derangement of Th1/Th2 differentiation. IL-2–mediated STAT5 activation and upregulation of GATA-3 expression is essential for the early Th2 commitment, independently of the IL-4/STAT6 signaling cascade (2, 31). To investigate whether FURIN regulates fate-determining transcription factors during T cell development, we measured thymic expression of GATA-3 and T-bet, and found that FURIN deficiency did not significantly affect the expression levels of either factor in single-positive CD4+CD8− cells (Supplemental Fig. 3). Collectively, these data suggest that the FURIN deficiency causes Th2 hyperpolarization by regulating the Th2 phenotype amplification phase through the IL-4/STAT6 route rather than by disrupting the normal thymic development or downmodulating the initial TCR signal transduction.

To test directly whether the skewed Th2 phenotype of FURIN cKO cells was due to cytokine signaling, we inhibited the IL-4/STAT6 pathway by adding IL-4 neutralizing Ab (2 μg/ml) into serum-free CD4+CD62L+ T cell cultures and measured the mRNA expression of GATA-3. Blocking IL-4 function prevented the GATA-3 overexpression in vitro activated FURIN cKO T cells (Fig. 4B). Interestingly, when the expression of IL-4Rα was analyzed, we observed that FURIN did not regulate Il4ra mRNA levels (Fig. 4C) but significantly inhibited the IL-4Rα protein expression on the naive CD4+ T cell surface presumably through a posttranslational mechanism (Fig. 4D). To verify the biological relevance of the increased IL-4Rα surface expression, we measured the IL-4–induced STAT6 activation by flow cytometry. As expected, naive CD4+CD62L+ T cells from FURIN cKO mice repeatedly showed more pSTAT6 after a short IL-4 stimulus when compared with littermate control mice (Fig. 4E). In conclusion, we show that the lack of FURIN upregulates the expression of IL-4Rα on naive Th cells, which then contributes to sensitized IL-4/STAT6 signal transduction and a shift in Th1/Th2 balance.

**FURIN interacts with RAC/DOCK2 and regulates T cell cytoskeleton dynamics**

To find a potential mechanistic explanation for the FURIN-dependent dysregulation of IL-4Rα and consequent Th2 bias, we sought to identify novel FURIN-interacting proteins in T cells.

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**FIGURE 4.** FURIN inhibits IL-4Rα cell-surface expression and IL-4–induced STAT6 phosphorylation. (A) CD4+ T cells were isolated from CD4cre-furf/f (cKO) and littermate control (wt) mice, and stimulated with soluble anti-CD3 (10 μg/ml) for indicated times. Total ERK and pERK were detected with Western blot, and pERK/ERK ratio was determined by measuring the intensity of the bands. Ratio from unstimulated wt sample was arbitrarily set as 1. Experiment was repeated twice with similar results. (B) Naive CD4+CD62L+ T cells from FURIN cKO and wt mice were cultured in serum-free media for 72 h in the presence of plate-bound anti-CD3 (10+10 μg/ml) activation and neutralizing anti–IL-4 Ab (2 μg/ml) as indicated. GATA-3 and housekeeping gene 18s expressions were determined by Q-RT-PCR, and relative expression in untreated wt sample was arbitrarily set as 1. Experiment was repeated twice with identical results. Error bars indicate SEM. (C) mRNA expression of Il4ra and 18s housekeeping gene was measured from steady-state naive CD4+CD62L+ T cells from FURIN cKO (n = 4) and littermate control (n = 3) mice. wt cell population was arbitrarily set as 1, and no statistical differences (ns) were observed between the groups (Mann–Whitney U test). Error bars indicate SEM. (D) Naive CD4+CD62L+ T cells from FURIN cKO and wt mice were cultured in serum-free media for 72 h in the presence of plate-bound anti-CD3/28 (10+10 μg/ml) activation and neutralizing anti–IL-4 Ab (2 μg/ml) as indicated. GATA-3 and housekeeping gene 18s expressions were determined by Q-RT-PCR, and relative expression in untreated wt sample was arbitrarily set as 1. Experiment was repeated twice with identical results. Error bars indicate SEM. (E) Phosphorylation of STAT6 was detected in CD4+CD62L+ T cells from FURIN cKO and littermate control mice. Cells were activated with IL-4 (1 or 10 ng/ml) for 15 min, and pSTAT6 was stained for FACS analysis. Geometric MFI values for pSTAT6: unstained 22, 26; wt 68, 85; and FURIN cKO 111, 121 (IL-4: 1, 10 ng/ml, respectively). Shown is a representative experiment of two performed.
FIGURE 5. FURIN interacts with RAC/DOCK2 complex and regulates F-actin polymerization. Jurkat E6-1 T cell lines stably expressing either Strep-tagged human wt FURIN (pcDNA3.1-hFURINwt-StrepIII) or enzymatically inactive (D153A) mut (pcDNA3.1-hFURINmut-StrepIII) were generated as described in Materials and Methods. Cell membrane fractions were isolated, and FURIN as well as associated proteins were affinity purified from lysates with Strep-tag columns. (A) Eluted proteins were separated by one-dimensional SDS-PAGE gel and visualized by silver staining. (B) A 20-kDa band, the intensity of which was higher in the FURINwt-containing cell elutes, was cut from the gel, and after enzymatic protein digestion and extraction from the gel, peptides were identified by MS (Proteomics Facility, Turku Centre for Biotechnology); shown is a list of 12 potential FURIN interaction partners. (C) Peptide sequence coverages for FURINwt-specific interaction candidate RAC isoforms 1–3 are shown. HeLa cells were transiently transfected with pcDNA3.1-hFURINwt-MYC, pcDNA-RAC1-V5, pCI-DOCK2-FLAG encoding constructs. (D) Colocalization of RAC1 and FURIN was detected with immunofluorescence using anti-MYC and anti-V5 Abs and DAPI staining. Representative image is shown; arrows identify (Figure legend continues)
To this end, we first generated human Jurkat E6-1 T cell lines that stably expressed either wt or enzymatically inactive mut (32) human FURIN-Strep-Tag fusion proteins. FURIN interactomes were purified from cell membrane fractions using Strep-tag affinity purification and SDS-PAGE gels followed by peptide identification with MS (33). Analysis of a 20-kDa band, the intensity of which was higher in the wt FURIN containing elutes, resulted in the identification of 12 potential interaction partners for FURIN (Fig. 5A–C). Of these, only small GTPase RAC1–3 fulfilled the identification criteria (i.e., correct m.w., sufficient sequence coverage, two or more identified peptides) and was specifically present in lysates containing the enzymatically active FURIN. Of note, RAC has been reported to directly promote Th1 polarization by upregulating IFN-γ (34), and together with its activator DOCK2, RAC is also directly implicated in the regulation of IL-4Rα recycling and degradation in CD4+ T cells (35, 36).

First, to validate our MS finding, we transfected HeLa cells with FURIN and RAC1 or DOCK2 encoding cDNAs. RAC1 and DOCK2 were found to be colocalized (Fig. 5D), and the association of FURIN and DOCK2 proteins was detected by coimmunoprecipitation (Fig. 5E), which collectively indicates the existence of FURIN/RAC1/DOCK2 complexes in the cells. Because the RAC/DOCK2 complex regulates the T cell cytoskeleton dynamics (36), we then investigated whether FURIN can also promote F-actin polymerization. The stable overexpression of wt FURIN significantly enhanced the level of F-actin polymerization in Jurkat T cells at both steady-state and in response to chemotactic signal SDF-1α (Fig. 5F). Taken together, our data show that FURIN interacts with RAC/DOCK2 complex and positively regulates the F-actin polymerization in T cells, which has previously been linked with downregulation of IL-4Rα on Th cell surface and inhibition of Th2 type immunity in vivo (34, 36, 37).

Discussion

Although the proprotein convertase FURIN has been shown to critically regulate diverse functions in homeostasis and pathology (9), its role in the T cell–mediated immune regulation is incompletely understood. Our initial studies revealed that FURIN is highly expressed in human Th1 type cells, where it promotes IFN-γ production (5). However, mice lacking T cell–expressed FURIN surprisingly presented with overly activated Th1 and Th2 cells, and inappropriate Treg function (18), which implied that FURIN has a multifaceted role in the CD4+ T cell biology. In this study, when mice were infected with a prototypic Th1 pathogen *T. gondii*, FURIN was found to be upregulated in CD4+ T cells in an IL-12–dependent manner. T cell–expressed FURIN was shown to be essential for host defense against the parasitic infection and the generation of Ag-specific Th1 and Th1–IL-10 cell responses. Activated FURIN cKO CD4+ cells showed propensity to polarize toward Th2 phenotype in vitro, which was accompanied with elevated cell-surface IL-4Rα expression on naive Th cells. Finally, exploring the FURIN interactome using Strep-tag purification and proteomics resulted in identification of cytoskeleton modifying RAC/DOCK2 complex as a novel FURIN interaction partner. FURIN promoted actin polymerization in T cells, which has previously been shown to mediate IL-4Rα internalization. Altogether, our results unravel an IL-12/TGF-β1–induced protease FURIN as a central regulator of Th cell polarization and demonstrate its criticality in cell-mediated immune responses.

Transcriptional profiling studies on IL-12 target genes have offered mechanistic insights into Th1 differentiation. Cytokine receptors like IL-2Rα, IL-12Rβ2, and IL-18Rα, TPL2 kinase, and transcription factors such as IFN regulatory factor 1 are all induced by IL-12, and are required for optimal IFN-γ production and cell-mediated immunity (5, 24). In this study, we showed that IL-12 is important for FURIN upregulation upon *T. gondii* infection in vivo, which suggested a regulatory role for this protease in host defense against intracellular pathogens. FURIN is thought to be widely expressed, and it autoregulates its own enzymatic activity (8). Thus, the expression levels of FURIN serve as a critical determinant for its substrate processing activity also during an immune response. Recent findings show that not only IL-12 and TCR signals in T cells, but also LPS stimulation in macrophages can upregulate FURIN (10, 38). FURIN is also secreted from cells upon immune activation, and increased serum levels were recently found in chronic typhoid carriers (39). Assessing serum FURIN levels has thus potential to become a future biomarker in detecting the activation of immune systems in host.

FURIN has an essential function in proteolytic processing of the inactive pro–TGF-β1 precursor into its bioactive form (40, 41). Consequently, CD4cre-furf/f mice largely phenocopy the immunological abnormalities (e.g., age-related systemic autoimmunity) seen in CD4cre-tgfbf/b mice (18, 42). However, FURIN-deficient Tregs were partly functional, and FURIN EKO effector CD4+ cells appeared more resistant to the suppressive activity of wt Tregs (18) than what was reported in TGF-β1 deficiency, which implies the existence of additional targets for FURIN in T cells. PCSK substrate molecules and interaction candidates have been investigated by a variety of methods, including gene expression correlations, peptide sequencing, and microarrays (43–45). In this study, we used Strep-tag affinity purification and MS to identify T cell–specific FURIN interactions (33). By using both wt and inactive mut forms of FURIN, we could distinguish proteins that specifically interact with the active FURIN enzyme in human Jurkat T cells. Although we carefully optimized the cell lysis protocols to catch FURIN protein efficiently, it is possible that the relatively high stringency that was required for effective lysis may have resulted in loss of some transient interaction partners. In our experiments, the FURIN interacting proteins were first separated in one-dimensional SDS-PAGE gel, and identifications were focused on the silver-stained bands with different intensities, which may obviously limit the number of identifiable interaction partners.

The identified novel FURIN-associated proteins, small GTPase RAC and its activator DOCK2, have diverse roles in T cell biology. In peripheral T cells, RACs are essential for TCR signaling (46) and reactivation-induced apoptosis (47), but RAC can also promote Th1 polarization by upregulating signaling via p38 and NF-κB pathways and IFN-γ production (34). At the molecular level, the divergent functions of the RAC/DOCK2 complex are mediated through the modulation of actin rearrangements and associated with T cell cytoskeleton dynamics (48). Indeed, it has been previously described that FURIN regulates the actin cytoskeleton dynamics by processing integrins in vascular smooth muscle cells.
produced STAT5 phosphorylation in CD4+ cells was intact (Fig. 4C).

The cytokine-modifying activity of RAC/DOCK2 complex regulates the IL-4Rα trafficking and its surface expression on activated T cells, which then contributes to the strength of IL-4/STAT6 signaling and ensuing Th2 polarization (35, 36). Our flow-cytometric analyses showed that FURIN deficiency significantly upregulates IL-4Rα protein levels on naive CD4+ T cells, which inherently express only low levels of this receptor chain (31). Accordingly, FURIN-deficient CD4+ T cells mimicked the phenotype of DOCK2 KOs by showing elevated Th2 responses, such as high serum IgE level, but in contrast with DOCK2-deficient animals (36), the Ag-specific IFN-γ response to chronic intracellular parasite infection was reduced in FURIN cKO mice. The ll4ra mRNA expression in CD4+ cells is initially primed by IL-2–induced STAT5 activation (31). FURIN-deficient Th cells showed unchanged ll4ra and Il2ra mRNA expressions, and the IL-2–induced STAT5 phosphorylation in CD4+ cells was intact (Fig. 4C and data not shown). In contrast, neutralizing IL-4 effectively blocked the excessive GATA-3 expression in FURIN cKO T cells, and the IL-4–induced STAT6 activation was sensitized. Collectively, our results thus suggest that FURIN regulates Th1/Th2 balance by coordinating cytokine dynamics and the IL-4Rα expression on the cell surface, which is essential for cytokine-dependent amplification of Th2 type cells. However, it is possible that by promoting RAC activity, FURIN enhances also directly IFN-γ and associated Th1 polarization (34). Further studies using endogenously expressed proteins and primary T cells are clearly required to decode the physiological molecular mechanisms by which the FURIN-mediated RAC activation regulates Th cell balance.

Cell-mediated immune responses are essential for host control over intracellular pathogens, such as Mycobacteria, Toxoplasma, Leishmania, and certain viruses. CD4cre-fur101 mice succumbed to T. gondii infection, arguing for the criticality of T cell–expressed FURIN in the generation of protective immunity. In the acute phase of T. gondii infection, FURIN was dispensable for survival, generation of Th1 cells, and normal serum cytokine responses. The early Th1 cell generation has been shown to depend on high levels of APC-produced IL-12 (21), which functions FURIN independently. However, increased T. gondii burdens in the brains of Cd4cre-fur101 mice demonstrated that FURIN has a T cell–intrinsic role in controlling parasite growth. The analysis of Ag-specific Th responses showed that FURIN regulated the generation of sustainable protective (Th1) and tolerogenic (Th1–IL-10+) cell populations. An analogous dual role has previously been reported for IL-27, which was initially characterized as a driver of Th1 type responses (50) and later shown to support suppressive IL-10–producing Tregs in T. gondii infection (51). In contrast, the lack of FURIN in T cells did not appear to promote the generation of single-positive CD4*IL-10+ cells in T. gondii–infected mice. These data could indicate that in the presence of elevated parasite burdens, FURIN deficiency is not able to contribute to the complementary induction of Th2 polarization. Importantly, a direct measurement of IL-4– and other Th2 cytokines postinfection would be required to clarify this aspect. Also, cytotoxic CD8+ T cells critically restrict T. gondii in the chronic phase, and the exhaustion of CD8+ T cells leads to uncontrollable parasite growth and encephalitis (52). Notably, in recall assays, we used STAg to induce T cell responses, which activates CD4+ cells through MHC class II. However, the role of FURIN in the regulation of CD8+ T cell biology is currently unknown, and we can thus not rule out the fact that T. gondii–infected CD4cre-fur101 mice could die because of impaired CD8+ T cell function. Importantly, our previous data demonstrate that FURIN deficiency accelerates T cell responses (18), which, in theory, can promote the exhaustion of CD8+ T cell responses in the brain.

Inhibiting the PCSK function has a potential to become a future means to treat immune-mediated diseases such as cancers, atherosclerosis, and infections (10–12). In addition, administering recombinant FURIN can alleviate the overactive immune cells in autoimmune diseases (11). Understanding how FURIN regulates cell-mediated immunity and Th balance is thus critical when such treatments are considered for clinical use. Our earlier results demonstrate that deleting FURIN activates T cells, which could be beneficial in treatment of cancers, for example. However, our results show that T cell–expressed FURIN is critical for host resistance to T. gondii and inhibits Th2 polarization. Therefore, interfering with the PCSK activity in patients may compromise protection against intracellular pathogens and result in allergic responses.

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Disclosures

The authors have no financial conflicts of interest.

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