



The Journal of Immunology

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of April 8, 2010

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J. Immunol. 2010;184:4488-4496; originally published
online Mar 10, 2010;

doi:10.4049/jimmunol.0903115

<http://www.jimmunol.org/cgi/content/full/184/8/4488>

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Uncoordinated 119 Preferentially Induces Th2 Differentiation and Promotes the Development of Asthma

Magdalena M. Gorska,^{*,†} Nicolas Goplen,^{*} Qiaoling Liang,^{*} and Rafeul Alam^{*,†}

The Th2 bias is a hallmark of allergic diseases. In this study, we show that the Th1 versus Th2 balance and the development of allergic asthma are strongly affected by the signaling protein uncoordinated 119 (Unc119). The expression of this adaptor protein is significantly increased in Th2 cells. Unc119 activates the Src family and inhibits the Abl family of tyrosine kinases. The activated Src family kinase Lck stimulates the activity of Itk and the expression of the transcription factor JunB. As a result, Unc119 promotes IL-4 production. Through inhibition of Abl kinases, Unc119 dampens IFN- γ production. Using adoptive transfer of Unc119-knockdown CD4 T cells, we show a critical role for Unc119 in the development of eosinophilic inflammation of airways, mucus production, and bronchial hyperreactivity in a mouse model. Intriguingly, the expression of the Unc119 protein is enhanced in CD4 T cells from patients with asthma. We speculate that the heightened expression of Unc119 promotes Th2, inhibits Th1 differentiation, and contributes to the pathogenesis of asthma in humans. *The Journal of Immunology*, 2010, 184: 4488–4496.

Asthma is a chronic airway disease that is characterized by bronchial hyperresponsiveness and airflow obstruction. There are 300 million people worldwide with asthma, and the prevalence of the disease is increasing (1). Chronic airway inflammation is fundamental to asthma pathogenesis. The seminal event in the development of inflammation in allergic asthma is the generation of the Th2 cells as a result of exposure to allergens (2). Naive CD4 T cells differentiate into Th2 cells upon receiving coordinate signals from TCR and IL-4R (3). Although much is known about signals stemming from IL-4R, the Th2-promoting TCR pathways are not fully understood. TCR does not have intrinsic enzymatic activity and relies on nonreceptor tyrosine kinases for signal generation (4, 5). Tyrosine kinases of Src and Tec families are known to play an important role (6, 7). There are two TCR-linked Src family members Lck and Fyn. The Tec family includes Txk and Itk. Lck and Itk have been shown to favor Th2 development, whereas Fyn and Txk support the differentiation of Th1 cells (6–9). A recent study indicates that tyrosine kinases of the Abl family, Abl and Arg, are critical for IFN- γ production, but redundant for IL-4 synthesis in nonpolarized T cells (10).

We and others cloned the adaptor protein uncoordinated 119 (Unc119) (11–13). We subsequently showed that Unc119 controls the constitutive transport of Lck to the plasma membrane via endosomes. Unc119 is also critical for stimulation of the Lck

enzymatic activity upon TCR triggering (14, 15). The effect of Unc119 on Fyn is short-lasting and of significantly lower magnitude (14, 16). Unc119 is required only for an initial short burst of Fyn kinase activity, but has no impact on Fyn activation during later stages of cell stimulation. Furthermore, Unc119 has no effect on the subcellular distribution of Fyn (15). In addition, we have recently reported that Unc119 is a potent inhibitor of Abl family kinases (17). Thus, Unc119 differentially affects three tyrosine kinases that are known to influence Th cell differentiation. In this study, we explore the role of Unc119 in Th1 and Th2 differentiation and in the pathogenesis of allergic asthma.

Materials and Methods

Abs

Rabbit polyclonal anti-Abl, anti-Arg, anti-CrkL, anti-JunD, goat polyclonal anti-Itk and anti-actin, mouse monoclonal anti-GATA3 (clone HG3-31), anti-T-bet (clone 4B10), anti-JunB (clone C-11) Abs were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-GFP Ab was from Invitrogen (Carlsbad, CA). Mouse monoclonal anti-Crk (clone 22/Crk) was acquired from BD Biosciences (San Jose, CA). Rabbit polyclonal anti-phospho-Crk (Tyr221) Ab was acquired from Cell Signaling Technology (Danvers, MA). The generation of the rabbit polyclonal anti-Unc119 Ab and the affinity purification was described previously (11, 14, 15, 17). For cell culture, the following Abs were used: Armenian hamster monoclonal anti-mouse CD3 (clone 145-2C11), Syrian hamster monoclonal anti-mouse CD28 (clone 37.51), mouse monoclonal anti-human CD3 (clone OKT3), mouse monoclonal anti-human CD28 (clone CD28.2), rat monoclonal anti-mouse IL-12/IL-23 (p40 subunit; clone C17.8), rat monoclonal anti-mouse IFN- γ (clone XMG1.2; all from eBioscience, San Diego, CA), and rat monoclonal anti-mouse IL-4 (clone 11B.11; National Cancer Institute Preclinical Repository).

Subject population

Allergic asthmatics, subjects with rheumatoid arthritis (RA) or allergic rhinitis subjects without asthma and without RA and healthy controls were recruited in accordance to a protocol approved by an institutional review board. The asthmatic population ($n = 11$; 23–59 y old) was characterized by a positive skin test for common allergens and reversible bronchoconstriction as demonstrated by an increase in FEV1 of at least 12% after a short-acting bronchodilator and/or positive methacholine test (PC20 for methacholine ≤ 8 mg/ml). Severity of asthma ranged from mild intermittent (FEV1 $> 80\%$) to severe persistent (FEV1 $< 60\%$) per the *National Asthma Education Expert Panel Guidelines*. Patients with asthma maintained their routine medications, which included inhaled steroids with or without long-acting bronchodilators, rescue bronchodilators, and/or leukotriene receptor antagonists. None were given systemic steroids, theophylline, or omalizumab. Patients with allergic rhinitis without

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Received for publication September 22, 2009. Accepted for publication February 2, 2010.

This work was supported by National Institutes of Health Grants RO1 AI0059719 and RO1 AI68088 (to R.A. and M.M.G.) and PPG HL36577 and N01 HHSN 272200700048C (to R.A.).

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Abbreviations used in this paper: BM, basement membrane; Contr, retrovirus encoding GFP alone; Eos, eosinophil; Lymph, lymphocyte; Mac, macrophage; MFI, mean fluorescence intensity; Neutr, neutrophil; RA, rheumatoid arthritis; sal, saline; sh, short hairpin; shC, control shRNA; shUnc119, shRNA for Unc119; Unc119, uncoordinated 119; WB, Western blot.

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asthma ($n = 10$; 18–59 y old) were recruited on the basis of a positive skin test and symptoms of allergic rhinitis with or without conjunctivitis, but not asthma. These patients continued their topical (nasal) medications, but withheld antihistamines for 48 h. None were given allergen immunotherapy. Patients with RA ($n = 7$; 36–65 y old) visited our clinic because of accompanying rhinitis (allergic and/or nonallergic). RA was diagnosed previously according to guidelines of the American Rheumatism Association. Patients with RA maintained their routine medications, which included a nonsteroidal anti-inflammatory drug as needed, celecoxib, or hydroxychloroquine. None were given biologics, methotrexate, or systemic steroids. Healthy subjects ($n = 11$; 20–57 y old) had no major medical history, including history of asthma, allergic rhinitis, RA, and negative skin prick tests for common allergens. Exclusion criteria for all subjects were: respiratory and other infections within the last 1 mo, immunodeficiency, use of immunosuppressants, pregnancy, participation in a clinical trial within the last 3 mo, and cigarette smoking.

Isolation and stimulation of human CD4 T cells

Mononuclear cells were isolated from the peripheral blood of healthy volunteers using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) as described by the reagent manufacturer. CD4 T cells were purified from mononuclear cells by negative selection using the human CD4⁺ T Cell Isolation Kit II (Miltenyi Biotech, Auburn, CA). CD4 T cells were stimulated with plastic-bound anti-CD3 and anti-CD28 Abs (2 μ g/ml of each Ab in the plate-coating buffer-PBS) or left unstimulated in RPMI medium containing 10% FBS for 24 or 72 h. Cells were lysed in the modified RIPA buffer (14).

Immunoprecipitation and Western blotting

Immunoprecipitation, PAGE, protein transfer to polyvinylidene difluoride membranes and Western blotting were performed as explained elsewhere (14).

Immune complex kinase assay

Mouse CD4 T cells were stimulated with an anti-CD3 Ab and an anti-Armenian hamster IgG secondary Ab as described (18). Cells were lysed in the modified RIPA buffer (14). Itk was isolated from cell lysates by immunoprecipitation using anti-Itk Ab. The immunoprecipitated Itk was incubated in the kinase buffer (100 mM HEPES, pH 7.4, 50 mM NaCl, 10 mM MgCl₂, 100 μ M Na₃VO₄, 500 μ M DTT, 25 mM β -glycophosphate) containing an additional 5 μ Ci ³²P-ATP (GE Healthcare, Piscataway, NJ), 2.5 μ M ATP, and the tyrosine kinase substrate-enolase (Sigma-Aldrich) at 5 μ M at 30°C for 5–10 min. Samples were run on polyacrylamide gels, transferred, and autoradiographed (14).

Th cell differentiation and measurement of cytokine release by ELISA

CD4 T cells were isolated from C57/BL6 splenocyte suspensions by negative selection using the mouse CD4 T cell Isolation Kit (Miltenyi Biotech). CD4 T cell differentiation into Th1 and Th2 cells was performed according to the well-established protocol (19, 20). On day 7, cells were washed and stimulated with PMA (50 ng/ml) and ionomycin (1 μ M) for 16 h. In some experiments, imatinib mesylate (LC Laboratories, Woburn, MA) at 10 μ M was added to cultures. The concentration of cytokines in the culture supernatants was measured by ELISA according to the manufacturer (BD Biosciences) protocol. Procedures on mice were approved by the Institutional Animal Care and Use Committee at National Jewish Health.

RNAi, plasmids, and retroviral infection of T cells

shRNAs for mouse Unc119 and luciferase (control) were designed and cloned into the retroviral vector Banshee-GFP as described (15). Mouse Unc119 cDNA was cloned into the bicistronic retroviral vector GFP-RV (21) as explained elsewhere (14, 17). CD4 T cells were infected with retroviruses at 36 and 60 h after initiation of culture, according to well-established protocols (14, 15).

Mouse model of acute asthma

OVA sensitization and challenge of mice was performed as described (22). At 48 h after the last challenge, lungs were removed and CD4 T cells were isolated as previously explained (23). Lung pieces were digested with collagenase. Recovered cells were washed, and CD4 T cells were isolated by positive selection using CD4 MicroBeads (Miltenyi Biotech). Lung CD4 T cells were examined for Unc119 expression by flow cytometry (see below). In a second set of experiments lungs, were lysed in the modified RIPA buffer for analysis of the Unc119 protein expression by Western blotting.

Flow cytometry

Purified human or mouse CD4 T cells were immunostained for Unc119 according to a previously described method (15). Stained cells were analyzed using the CyAn ADP cytometer (Beckman-Coulter, Fullerton, CA).

Adoptive transfer of shRNA-expressing CD4 T cells

CD4 T cells were isolated from splenocyte suspensions of OT-2 TCR transgenic mice (gift from Dr. Philippa Marrack, National Jewish Health, Denver, CO). T cells were activated with OVA peptide (aas 323–339) and irradiated C57BL/6 splenic APCs in Th2-skewing conditions (day 0) as described (24). Cells were infected with retrovirus for Unc119 shRNA or luciferase shRNA (36 and 60 h) and sorted by flow cytometry (selection marker-GFP). Sorted cells were expanded in cytokines for 2 d. On day 7, sorted cells or PBS were injected via a tail vein into C57BL/6 recipients (7×10^6 cells per mouse). Recipient mice were challenged intranasally with OVA (150 μ g in 15 μ l PBS) 24 h before and 24, 48, and 72 h after injection. Forty-eight hours after the last challenge, the airway reactivity to aerosolized methacholine was measured by the respiratory apparatus Flexivent (Scireq, Montreal, Quebec, Canada) (22), and bronchoalveolar lavage (BAL) was performed. Lungs were sectioned and stained with H&E, periodic acid and fluorescent Schiff's reagent or anti-GFP Ab and DAPI as explained elsewhere (22, 25). The morphometric analysis of stained sections was performed as described (22).

Statistical analysis

Statistical analyses in human studies were performed using Mann-Whitney *U* test. Statistical analyses in mouse studies were done by Student *t* test. Results are shown as mean $\pm$ SD. $p < 0.05$ was considered statistically significant.

Results

The expression of Unc119 is upregulated in CD4 T cells from asthmatic subjects and in the asthmatic lung

T cells play a central role in allergic inflammation, and Unc119 is important for their activation. To address the role of Unc119 in asthma, we first attempted to determine whether the expression of Unc119 is changed in T cells from allergic asthmatic subjects. Flow cytometric analysis showed an increased Unc119 protein level in peripheral blood CD4 T cells from allergic asthmatic subjects (Fig. 1A, 1B). The Unc119 protein expression in CD4 T cells from allergic patients without asthma was similar to that in healthy control subjects (Fig. 1A, 1B). The result indicates that more widespread Th2 inflammation is required for induction of Unc119 expression in CD4 T cells. The Unc119 protein level was normal in subjects with rheumatoid arthritis, which is a Th1/Th17-type disease (Fig. 1A, 1B). We also examined the expression of Unc119 in CD4 T cells isolated from the lung tissue from a mouse model of asthma. C57BL/6 mice were immunized and challenged with OVA. The expression of Unc119 was higher in lung CD4 T cells from the OVA-challenged mice compared with those from the saline-treated animals (Fig. 1C). The Unc119 level was also higher in the lung lysate from OVA-challenged mice (Fig. 1D).

The expression of Unc119 in human CD4 T cells is induced by prolonged TCR stimulation

We investigated the mechanism responsible for induction of Unc119. Because Unc119 impacts on TCR signaling, we asked whether TCR signaling could affect the Unc119 protein level. We studied peripheral blood CD4 T cells from healthy donors. Stimulation with the combination of anti-CD3 and anti-CD28 Abs resulted in upregulation of the Unc119 protein (Fig. 1E). Importantly, the induction of Unc119 required a prolonged TCR stimulation (72 h). The late induction of Unc119 is intriguing as it has been previously demonstrated that only prolonged TCR signals favor Th2 commitment (26). We also observed that a shorter incubation (24 h) with or without anti-CD3/anti-CD28 Abs actually resulted in a drop of Unc119 protein level below that seen in freshly purified CD4 T cells (Fig. 1E).

The Unc119 protein is upregulated in Th2 cells

We examined the expression of Unc119 in the course of Th1 and Th2 differentiation (Fig. 1F). At 24 h, Unc119 expression declined below the level detected in freshly isolated CD4 T cells. The

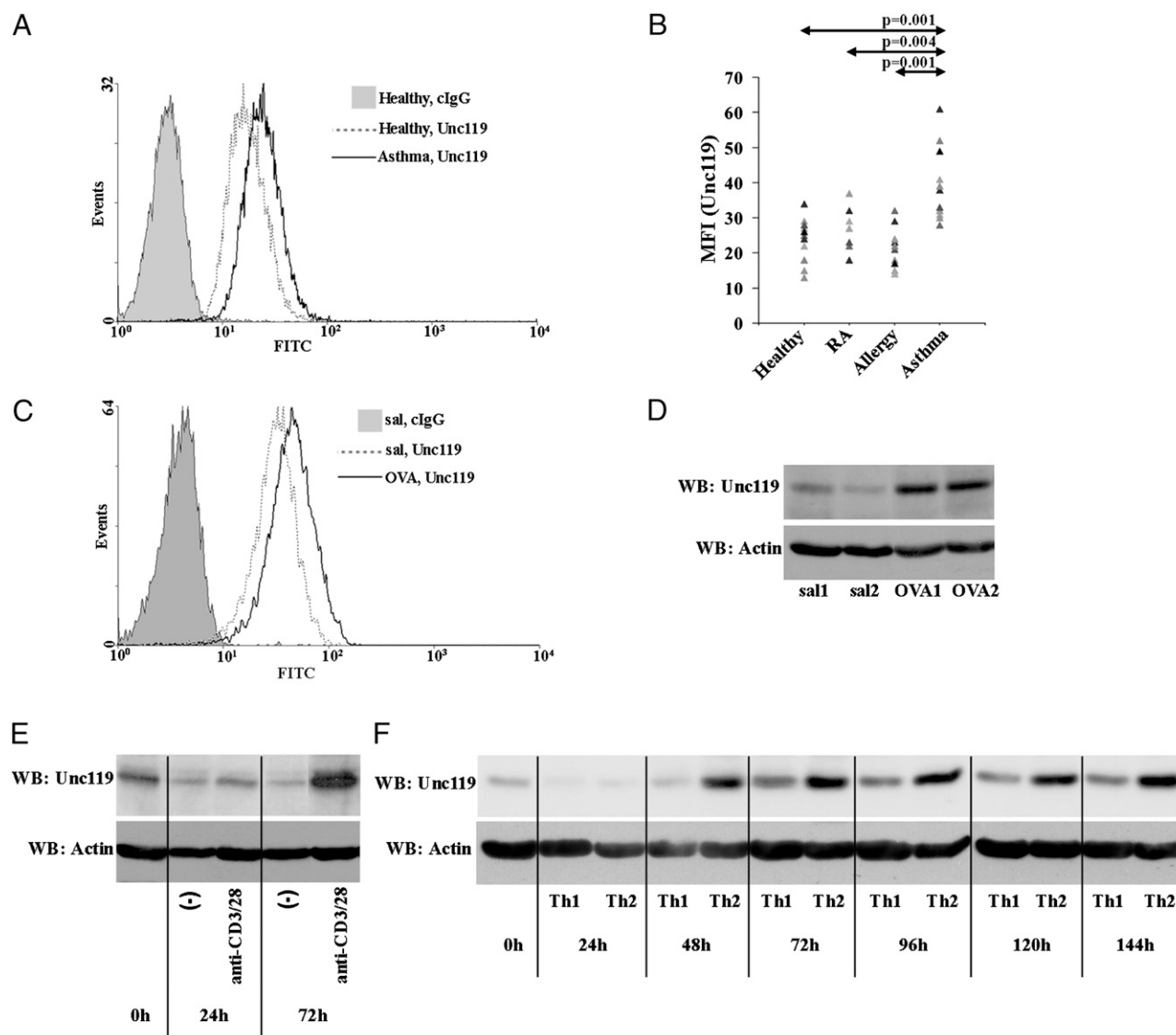


FIGURE 1. Expression of Unc119 in asthma. *A* and *B*, Flow cytometric analysis of the Unc119 protein expression in peripheral blood CD4 T cells from healthy subjects and subjects with RA, allergic rhinitis, or allergic asthma. The suitability of anti-Unc119 Ab for fluorescent immunostaining has been previously demonstrated (15). The affinity-purified anti-Unc119 Ab brightly stains normal T cells, but does not react with Unc119-knockdown T cells, which underscores the specificity of this Ab (15). An overlay histogram in *A* demonstrates results from a single healthy and a single asthmatic subject. *B*, The mean fluorescent intensity (MFI) of anti-Unc119 staining of CD4 T cells from 11 healthy subjects, 7 subjects with RA, 10 subjects with allergic rhinitis, and 11 subjects with allergic asthma. cIgG, staining with control Ig; Unc119, staining with the anti-Unc119 Ab. *C*, Flow cytometric analysis of Unc119 protein expression in lung CD4 T cells from the mouse model of asthma. Mice were immunized and challenged with OVA. Control mice received saline (sal). CD4 T cells were isolated from lungs as described (23), stained, and analyzed as in *A*. A representative overlay histogram is shown ($n = 3$). *D*, Western blot (WB) analysis of total lung lysates. Mice were treated as in *C*. Lung lysates were blotted with anti-Unc119 and anti-actin Abs. Figure is representative of three experiments from two different mice from each study group. *E*, Western blot analysis of CD4 T cells stimulated via the TCR. CD4 T cells from a healthy subject were stimulated with plate-bound anti-CD3 and anti-CD28 Abs or incubated in the media alone (–) for 0, 24, or 72 h. Cells were lysed and blotted with anti-Unc119 and anti-actin Abs ($n = 3$). *F*, Western blot analysis of Th1 and Th2 cell lysates. Mouse CD4 T cells were stimulated under Th1- or Th2-skewing conditions and harvested at 0, 24, 48, 72, 96, 120, and 144 h. Cells were lysed and lysates were blotted as in *E* ($n = 3$).

results are consistent with the data in Fig. 1*E*. At 48 h, Unc119 expression increased dramatically in Th2 cells and stayed at this highly elevated level throughout development. Unc119 was also re-induced in Th1 cells, but at a slower kinetics and to a significantly lower level than that observed in Th2 cells.

Unc119 has opposing effects on IL-4 and IFN- γ production

We examined the role of Unc119 in T helper cytokine production. The biologic relevance of a protein is best studied in a loss of function model. We previously demonstrated a substantial knockdown of Unc119 in primary mouse CD4 T cells by infection with a retrovirus encoding a short hairpin (sh) RNA (15). We also showed that Unc119 knockdown

did not affect the expression of Lck, CD3 ϵ , and CD4 (15). Therefore, to study the role of Unc119 in T helper cytokine production, mouse CD4 T cells were stimulated via TCR in Th1- or Th2-skewing conditions and, at 36 and 60 h, infected with the aforementioned retroviruses encoding GFP and shRNA for Unc119 or luciferase as a control. It is important to note that the retroviral infection requires prior cell activation. Subsequently, cells were sorted on the basis of the selection marker (i.e., GFP) expression. On day 7, sorted cells were washed and stimulated with PMA and ionomycin. Unc119 knockdown in Th2 cells resulted in 2.2- and 1.4-fold decreases in IL-4 and IL-5 production, respectively (Fig. 2*B*). Unc119 knockdown in Th1 cells led to a 2.3-fold increase in IFN- γ synthesis (Fig. 2*A*). Thus,

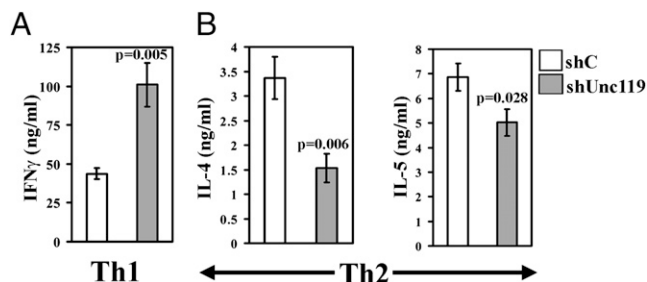


FIGURE 2. Effects of Unc119 knockdown on Th1 and Th2 cytokine production. *A* and *B*, Purified spleen CD4 T cells from C57BL/6 mice were stimulated with anti-CD3 and anti-CD28 Abs under Th1-skewing (*A*) or Th2-skewing (*B*) conditions. At 36 and 60 h, cells were infected with retroviruses encoding GFP and shRNA for Unc119 (shUnc119) or for luciferase (control shRNA [shC]) and sorted on the basis of GFP expression. On day 7, cells were washed and counted using trypan blue as a dead cell marker. The trypan blue-negative cells constituted $97 \pm 2\%$ of total cells. Accordingly, we previously reported that Unc119 did not significantly affect primary T cell survival (14). Equal numbers of cells were stimulated with PMA/ionomycin for 16 h. At the end of the culture, the trypan blue-negative cells were counted again. The absolute and relative numbers were similar in all cultures. The release of IFN- γ (Th1 cultures), IL-4, and IL-5 (Th2 cultures) was measured by ELISA ($n = 4$).

Unc119 promotes the release of Th2 cytokines, especially the release of IL-4, whereas it inhibits IFN- γ production.

Unc119 differentially affects Itk and Abl kinases

We attempted to examine the molecular mechanism that would link Unc119 to Th1/2 cytokine synthesis. As already mentioned, Unc119 activates the Th2-promoting kinase Lck (14, 15). Lck phosphorylates and activates the tyrosine kinase Itk, which substantially contributes to IL-4 gene transcription (6). We tested the effect of Unc119 on Itk enzymatic activity in an immune complex kinase assay. The activation of Itk in Unc119 knockdown and Unc119-overexpressing CD4 T cells was significantly impaired and augmented, respectively (Fig. 3*A*, 3*B*). Interestingly, Unc119 affected both basal and TCR-regulated Itk activity. Accordingly, we have previously observed that Unc119 influences the basal activity of Lck (14). Therefore, Unc119 is critical for activation of two Th2-promoting tyrosine kinases—Lck and Itk. We next investigated the molecular mechanism of increased IFN- γ secretion by Unc119-knockdown T cells. We previously showed that Unc119 bound and inhibited Abl family kinases in fibroblasts (17). Abl kinases have been reported to favor IFN- γ production (10). Unc119 interacted with Abl in resting T cells (Fig. 3*C*). The association remained the same in cells stimulated with an anti-CD3 Ab. Abl tyrosine kinases are activated by multiple extracellular and intracellular stimuli (e.g., cell-to-cell adhesion, growth factors, Ag, genotoxic stress) (27). Accordingly, Abl kinases show some activity even in the absence of TCR stimulation (28). We investigated the activation of Abl kinases using an Ab against the phosphorylated tyrosine 221 of the Abl-specific substrate—Crk (28). The phosphorylation of Crk was greatly augmented in Unc119-knockdown T cells (Fig. 3*D*). Our results indicate that Unc119 exerts a differential effect on Th1- and Th2-promoting tyrosine kinases in T cells.

Inhibition of Abl family kinases prevents excessive production of IFN- γ by Unc119-knockdown Th1 cells

Next, we asked whether Abl kinases were responsible for augmented IFN- γ release by Unc119-knockdown cells. To answer this question, we used the Abl kinase inhibitor imatinib mesylate (STI571, Gleevec) (10, 27, 28). CD4 T cells were activated under Th1 and Th2-skewing conditions, infected with shRNA-encoding retroviruses, and sorted as described previously. Imatinib or vehicle was added to cultures 48 h

after the last infection. The 48 h time point coincides with the highest GFP fluorescence and maximum Unc119 knockdown. Cells were cultured under the skewing conditions with or without imatinib for an additional 48 h. Next, cells were washed and stimulated with PMA and ionomycin in the presence or absence of imatinib for an additional 16 h. The concentration of cytokines in the culture supernatants was measured by ELISA. We observed 2.9- and 7.5-fold reductions in IFN- γ secretion by imatinib-treated control and imatinib-treated Unc119-knockdown Th1 cells, respectively (Fig. 3*E*). The amount of IFN- γ in supernatants from imatinib-treated Unc119-knockdown cells was comparable to that in supernatants from imatinib-treated control cells. Imatinib did not significantly affect the IL-4 production by Th2 cells. Imatinib-treated control and Unc119-knockdown Th2 cells tended to produce slightly less IL-4 than the vehicle-treated counterparts but the difference did not reach statistical significance (Fig. 3*F*). Abl family kinases, especially Abl, are known to affect cell proliferation and survival (10, 27, 29). Dependent on the experimental conditions, Abl has been reported to promote or inhibit apoptosis in other cell types (29). Thus, the ELISA cultures were carefully set to contain an equal amount of live (trypan blue-negative) cells. The amount of live cells at the conclusion of this 16 h PMA/ionomycin stimulation was the same in imatinib- and vehicle-treated cultures. Our results are in agreement with previous studies that demonstrated that T cell survival was not regulated by Abl kinases (10). Thus, the profound inhibition of IFN- γ release by imatinib cannot be attributed to the induction of cell death or to the inhibition of cell cycle. Next, we asked whether the diminished sensitivity of Th2 cells to imatinib was due to the absence of Abl kinases or its substrates, Crk and CrkL (27). The expression of Abl, Arg, and CrkL was equal in Th1 and Th2 control and Unc119-knockdown cells (Fig. 3*G*). Th1 cells demonstrated a slight reduction in Crk expression (Fig. 3*G*). Thus, the reduced sensitivity of Th2 cells to imatinib cannot be explained by lack of expression of its molecular targets.

Unc119 differentially affects the expression of T-bet and JunB transcriptional factors

We examined the effect of Unc119 on key Th1 and Th2 transcription factors. Unc119 knockdown in Th1 cells resulted in a moderate upregulation of T-bet expression (Fig. 3*H*). Unc119 knockdown led to a considerable reduction in the JunB level in Th2 cells, but it did not have any significant effect on the level of GATA3 (Fig. 3*I*, 3*J*).

Unc119 is important for the development of allergic asthma

Transfer of in vitro differentiated OVA-specific Th2 cells to non-immunized recipients followed by airway challenge with OVA results in substantial airway inflammation and increased airway reactivity to methacholine (30, 31). We asked whether decreased expression of Unc119 (by shRNA) in transferred T cells would impact on their ability to induce the aforementioned features of allergic asthma. We have used CD4 T cells from OT-2 TCR transgenic mice as donor cells. These T cells recognize OVA peptide (aa 323–339) in the context of I-A^b. CD4 OT-2 T cells were subjected to Th2-skewing conditions and infected twice at 36 and 60 h with the aforementioned retrovirus for shRNA for Unc119 or sh-luciferase. Cells were sorted by GFP expression and injected i.v. into C57BL/6 mice. Control mice received PBS (negative control). OVA was administered intranasally to recipient mice 24 h before injection, and the procedure was repeated 24, 48, and 72 h after the transfer. Mice were examined at 48 h after the last OVA challenge. Mice transferred with control (sh-luciferase) T cells had elevated amounts of Th2 cytokines in the BAL fluid (Fig. 5*D–F*) and developed eosinophilic inflammation of airways (Figs. 4*A*, 5*A–C*). In mice injected with Unc119-knockdown T cells, the concentration of Th2 cytokines and the numbers of infiltrating inflammatory cells in the lung were substantially reduced. In control

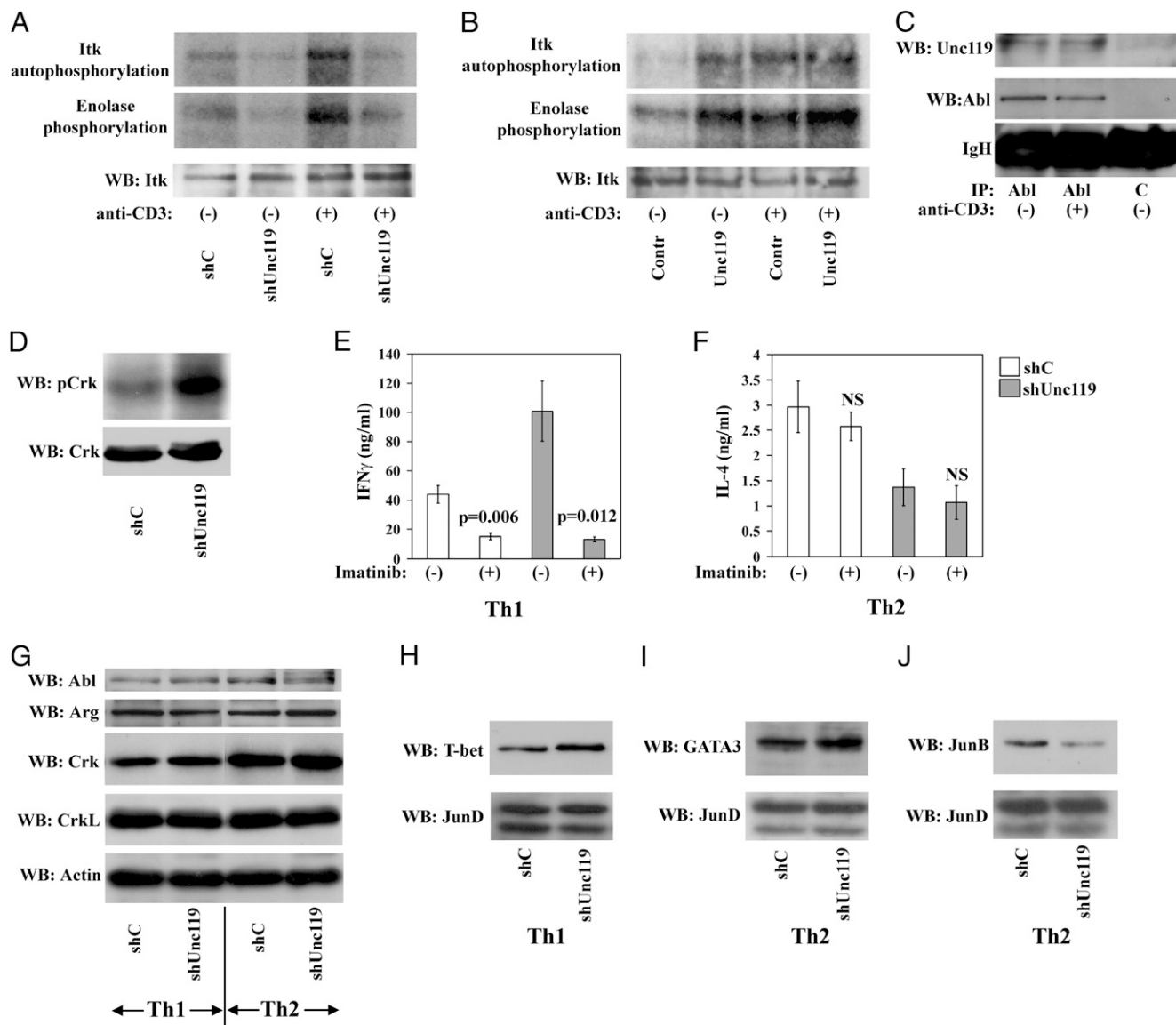
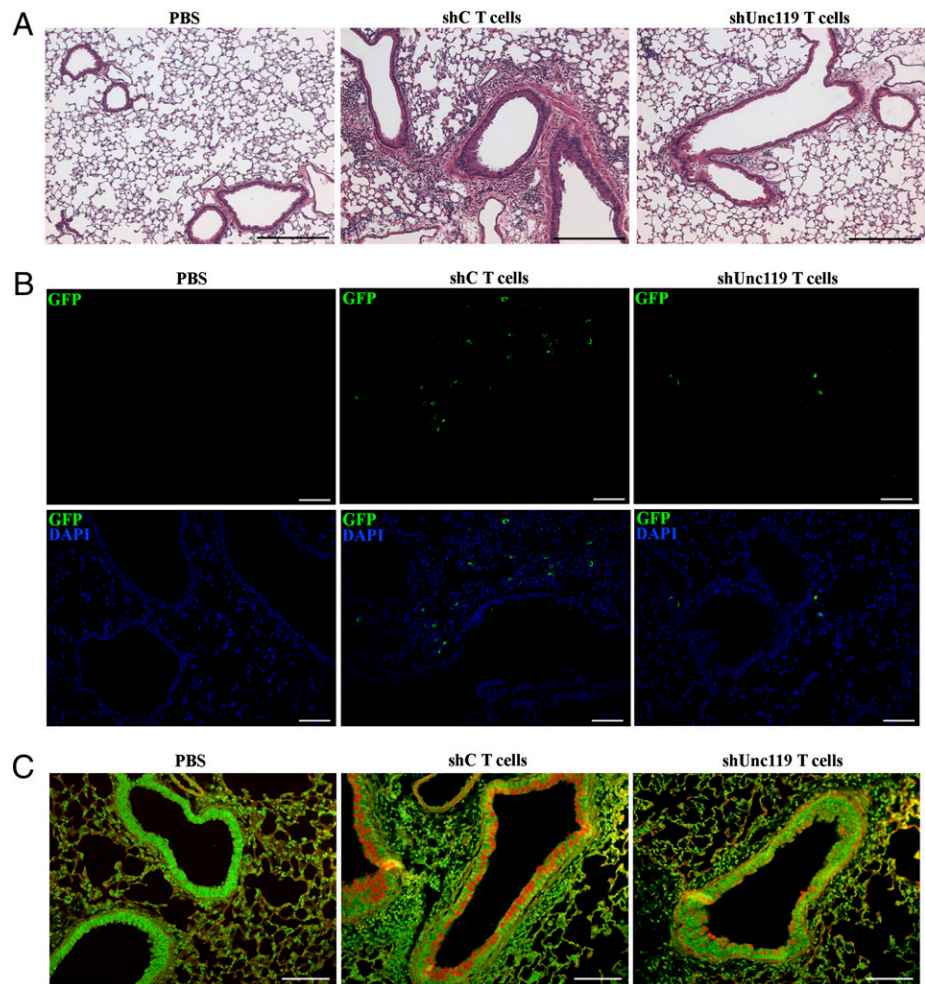


FIGURE 3. Effects of Unc119 on Th1- and Th2-promoting Abl family kinases and Itk, respectively. *A* and *B*, Purified spleen CD4 T cells were stimulated with anti-CD3 and anti-CD28 Abs and IL-2. At 36 and 60 h, cells were infected as in Fig. 2*A* (*A*) or with a retrovirus encoding GFP alone (Contr; *B*) or GFP and Unc119 protein (Unc119; *3B*). Cells were sorted as in Fig. 2*A*. After sorting, cells were cultured in IL-2 alone for 48 h. Next, the activation of Itk was examined. Cells were washed and incubated with a soluble anti-CD3 Ab (+) or control IgG (-) on ice for 30 min. Cells were washed and incubated with a soluble anti-Armenian hamster IgG secondary Ab in 37°C for 3 min (18). Cells were lysed. Lysates were immunoprecipitated with an anti-Itk Ab. Itk immunoprecipitates were incubated with ^{32} P-ATP, cold ATP, and the tyrosine kinase substrate (enolase). Reactions were run on polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and exposed to the radiolabelled film. Membranes were subsequently blotted with the anti-Itk Ab ($n = 3$). *C*, Jurkat CD4 T cells were stimulated with a plate-bound anti-CD3 Ab or incubated in the media alone (-) for 30 h. Cells were lysed. Lysates were subjected to immunoprecipitation (IP) with an anti-Abl Ab or control IgG (*C*). Immunoprecipitates were blotted with anti-Unc119 and anti-Abl Abs. *D*, Purified CD4 T cells were stimulated with anti-CD3/anti-CD28 Abs and IL-2, infected and sorted as in Fig. 2*A*. After sorting, cells were kept in media with IL-2 for 48 h. Next, cells were washed and lysed. Lysates were subjected to Western blotting with anti-phospho-Crk (Tyr 221; pCrk) and anti-Crk Abs ($n = 3$). *E* and *F*, CD4 T cells were stimulated in Th1/2 skewing conditions and infected as in Fig. 2*A* (*E*) and 2*B* (*F*). GFP-positive cells were sorted 48 h after the second infection and imatinib (+) or vehicle (-) was added to cultures. Cells were incubated with (+) or without (-) imatinib under Th1/2, skewing conditions for an additional 48 h. Next, the cells were washed, and the cell viability determined ($96 \pm 3\%$ of cells were trypan blue-negative). Cells were stimulated with PMA and ionomycin with (+) or without (-) imatinib for 16 h. At the conclusion of the culture, the relative numbers of trypan blue-negative cells were comparable in all cultures ($83 \pm 3\%$ of total cells). The absolute numbers of these cells were similar in all cultures as well. The concentration of cytokines in supernatants was determined by ELISA ($n = 3$). *G*, CD4 T cells were stimulated, infected and sorted as in Fig. 2*A* and 2*B*. On day 7, cells were lysed. The expression of Abl, Arg, Crk, CrkL, and actin proteins was assessed by Western blotting using respective Abs ($n = 3$). *H* and *I*, CD4 T cells were cultured in Th1- or Th2-skewing conditions, infected, sorted as in Fig. 2*A* and 2*B* (*H* and *I*, respectively), and nuclear extracts were prepared. Nuclear fractions were blotted with anti-T-bet (*H*) or anti-GATA3 (*I*) Abs. The protein loading was verified using an Ab against JunD, which is a constitutively expressed member of the AP-1 family ($n = 3$). *J*, Unc119-knockdown and control Th2 cells were stimulated with plate-bound anti-CD3 and anti-CD28 Abs for 16 h. Nuclear fractions were isolated and blotted with anti-JunB and anti-JunD Abs. We studied JunB in stimulated Th2 cells as resting cells showed a low level of JunB in nuclear fractions (not shown); $n = 3$.

experiments, we examined the lungs for the presence of transferred T cells by staining with an anti-GFP Ab. We detected a considerable number of GFP+ sh-luciferase expressing cells in control mouse lungs (Figs. 4*B*, 5*G*). The number of lung-infiltrating GFP+ Unc119-

knockdown T cells was reduced. This result is not surprising. First, owing to decreased Lck and Itk activities, Unc119-knockdown Th2 cells are likely to have some defects in migration. Second, the continuous influx of Unc119-knockdown T cells into airways might be

FIGURE 4. The role of CD4 T cell-expressed Unc119 in the development of allergic inflammation in the lung. **A** and **C**, Lung inflammation after adoptive transfer of Ag-specific Unc119 knockdown CD4 T cells. OT-2 CD4 T cells were activated with OVA peptide-pulsed irradiated C57BL/6 splenic APCs under Th2-skewing conditions. Cells were infected and sorted on the basis of GFP expression as in Fig. 2*B*. On day 7, sorted shUnc119-expressing T cells, shC-expressing T cells or PBS were injected i.v. into C57BL/6 recipients. Recipient mice were challenged intranasally with OVA 24 h before and 24, 48, and 72 h after the injection. The mice were examined 48 h after the last challenge. Lungs were sectioned and stained with H&E (**A**; scale bar, 100 μ m) or periodic acid and the fluorescent Schiff reagent (periodic acid and fluorescent Schiff's method) (25), which stains mucin in red and nucleic acids of cells in green (**C**; scale bar, 50 μ m). **B**, Presence of transferred T cells in the lung. Lungs sections prepared as in **A** were stained with an anti-GFP Ab and the nuclear dye DAPI. The top part of **B** contains fluorescent microscopy images of GFP alone. In the bottom part the GFP/DAPI overlay images are shown. Scale bar, 25 μ m; $n = 5$ mice per group.



prematurely terminated because of diminished recruitment or activation of other inflammatory cells (reduced strength of a positive feedback).

We next examined the mice for functional outcomes of airway inflammation. Control mice developed excessive airway mucus production (Figs. 4*C*, 5*H*, 5*I*) and increased airway reactivity to methacholine (Fig. 5*J*). In mice receiving Unc119-knockdown T cells, the aforementioned features of asthma were significantly diminished. Thus, we have demonstrated a critical role for Unc119 in the development of an experimental Th2 disease. A schematic diagram of the opposing effect of Unc119 on Th1 and Th2 differentiation is presented in Fig. 6.

Discussion

In this study, we uncover the role of the signaling molecule Unc119 in Th1/2 cytokine production in vitro and its relevance for allergic asthma in vivo. The expression of Unc119 is augmented in mouse Th2 cells and CD4 T cells from asthmatic subjects. Unc119 is required for the production of Th2 cytokines, especially for the production of IL-4. In contrast, it inhibits the release of IFN- γ . The molecular mechanism involves opposing effects of Unc119 on Th2-promoting tyrosine kinases Lck and Itk and Th1-promoting tyrosine kinases of the Abl family (Fig. 6). The T cell-expressed Unc119 is critical for development of allergic asthma as demonstrated in adoptive transfer experiments.

As discussed earlier, Unc119 is critical for the plasma membrane anchoring and the activation of the TCR-associated Src family tyrosine kinase Lck (14, 15). In transgenic mice, which express

dominant negative Lck, the development of Th2 cells is impaired whereas the maturation of Th1 cells is largely intact (7). Upon immunization, these mice are unable to produce IgE and IgG1 Abs, the two Ig isotypes that are driven by IL-4. In contrast, they retain full ability to produce IgG2a, an Ig that is stimulated by the Th1 cytokine IFN- γ . The Th2-promoting capacity of Lck can be attributed, at least in part, to the activation of Itk. Itk is an important substrate for Lck (32). Itk-deficient T cells have impaired ability to differentiate into IL-4-producing cells (6, 33). The mechanism involves diminished nuclear translocation of NFATc, reduced ERK activation and augmented T-bet expression. Interestingly, the expression of GATA3 is only slightly impaired (33). Itk-deficient mice do not mount Th2 responses in vivo as demonstrated in a parasite-infection model and in an allergic asthma model (6, 34). Thus, the impairment of Th2 cytokine production by Unc119-knockdown T cells, and the inability of these cells to induce experimental asthma in the adoptive transfer model, is likely caused by the diminished Lck and Itk activities.

We observed reduced nuclear expression of JunB in Unc119-knockdown Th2 cells. JunB is critical for Th2 differentiation (35–37). JunB expression is positively regulated by the ERK pathway (38). ERK activation is stimulated by Lck and Itk (33, 39). Previously, we reported severe impairment of ERK activity in T cells with reduced Unc119 expression (14). Thus, we believe that the diminished JunB expression in Unc119-knockdown cells is a result of decreased Lck and Itk activities. We did not detect any significant drop in the GATA3 level. This result is consistent with Itk knockout data (33).

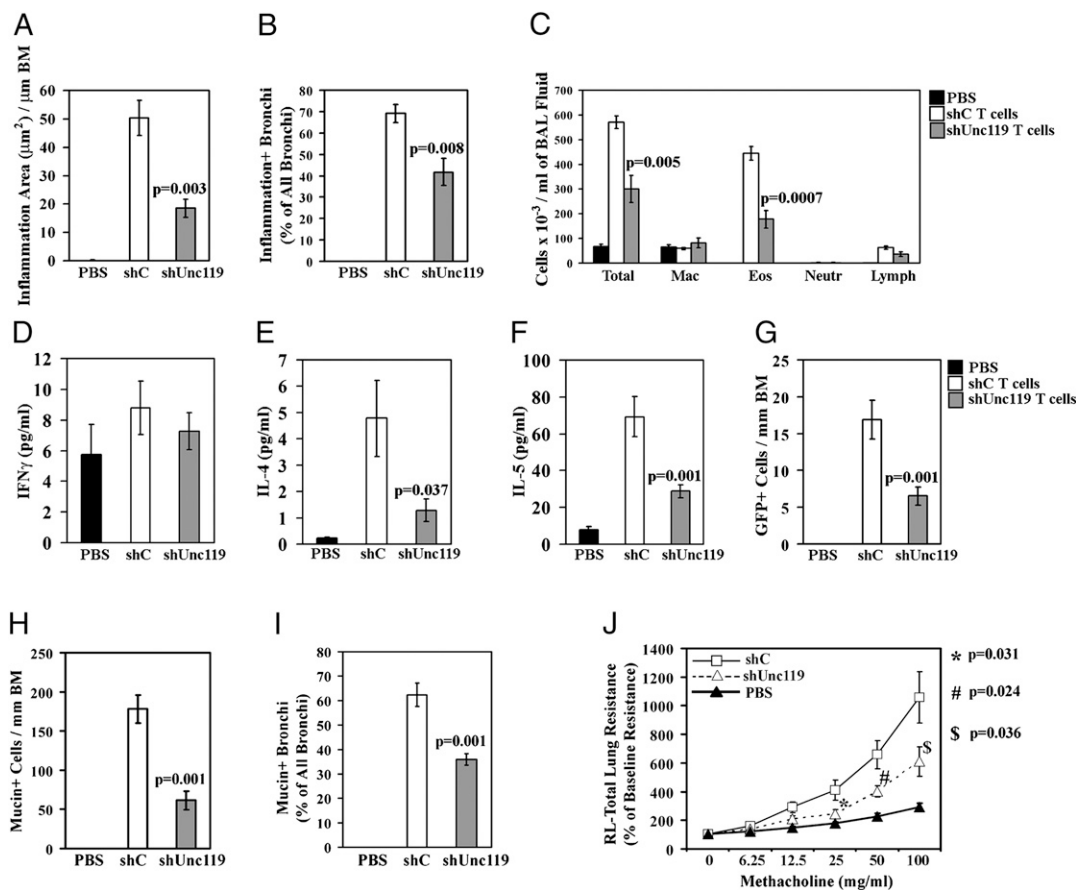


FIGURE 5. The role of CD4 T cell-expressed Unc119 in asthma. *A* and *B*, The quantification of airway inflammation in mice treated as in Fig. 4*A*. The mean area of inflammation per micrometer basement membrane (BM) of mid-sized bronchi (the BM perimeter: 300–1000 μm ; *A*) and the number of inflammation-positive (+) bronchi (*B*) were calculated ($n = 5$ mice per group). *C*, Cellular composition of the BAL fluid collected from mice adoptively-transferred as in Fig. 4*A*. The total BAL cell number and the number of macrophages (Mac), eosinophils (Eos), neutrophils (Neutr) and lymphocytes (Lymph) were determined ($n = 5$ mice per group). *D–F*, Production of T helper cytokines in lungs from adoptively transferred mice. The concentration of IFN- γ (*D*), IL-4 (*E*), and IL-5 (*F*) was measured in the BAL fluid by ELISA ($n = 5$). *G*, The quantification of transferred T cells in the lung. The number of GFP+ cells (see Fig. 4*B*) per millimeter BM of mid-sized bronchi was calculated ($n = 5$ mice per group). *H* and *I*, The fluorescent microscopy images of mucin staining (see Fig. 4*C*) were subjected to measurements. The number of mucin-positive (+) cells per millimeter BM of mid-sized bronchi (see Fig. 4*H*) and the number of mucin-positive (+) bronchi (see Fig. 4*I*) were determined ($n = 5$ mice per group). *J*, The bronchial reactivity to methacholine of adoptively transferred mice. The total lung resistance in response to increasing concentrations of aerosolized methacholine was measured using a Flexivent apparatus ($n = 5$ mice per group).

Unc119 regulates the Th1-promoting tyrosine kinases Abl, Arg and Fyn (this manuscript and Ref. 14, 16, 17). Unc119 constitutively associates with Abl and inhibits its enzymatic activity. Abl/Arg-double deficient T cells and Fyn-deficient T cells demonstrate selective impairment of IFN- γ production (9, 10). Abl kinases are critical for the activation of JNK in T cells (10). Abl kinases play much lesser role in the activation of ERK1/2 (10). Several reports indicate that JNK and ERK1/2 favor Th1 and Th2 development, respectively (40–42). Thus, the selective reduction in IFN- γ synthesis in Abl/Arg-double deficient T cells could be explained by the inhibition of the JNK pathway. Unc119 strongly inhibits Abl and Arg and moderately activates Fyn. Unc119 is important only for the early phase of Fyn activation. Unc119 knockdown T cells demonstrate augmentation of IFN- γ production. We believe that, in terms of IFN- γ synthesis, the effect of Unc119 on Abl kinases prevail over that on Fyn. We speculate that the IFN- γ production by Unc119 knockdown T cells is the result of hyperactivation of Abl family kinases and, possibly, an Unc119-independent late-phase Fyn activation (17). In addition, the diminished Itk activity may synergize in the stimulation of IFN- γ production through the induction of T-bet expression (33). However, the effect on Itk is not dominant, because the inhibition of Abl kinases by imatinib efficiently abrogates IFN- γ production in Unc119 knockdown T cells.

We observed a moderate elevation of T-bet in Unc119-knockdown Th1 cells. The effect of Itk on T-bet is well established (33). The role of Abl in the regulation of T-bet expression remains to be investigated. Abl may affect T-bet expression indirectly via

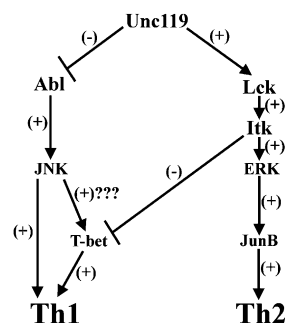


FIGURE 6. A proposed model of Th cell regulation by Unc119. Unc119 has opposing effects on Lck and Abl tyrosine kinases. Unc119 activates Lck and inhibits Abl family kinases. Consequently, Unc119 induces the phosphorylation of Itk kinase by Lck. Itk supports Th2 differentiation via positive regulation of ERK MAPK and the transcriptional factor JunB. Abl is essential for activation of JNK MAPK. JNK preferentially supports Th1 differentiation. The mechanism may involve T-bet.

activation of JNKs and their substrate c-Jun. c-Jun/ATF2 binding sites have been identified in the IFN- γ promoter (43). In addition, JNK2 regulates the expression of IL-12R β 2 (41).

The expression of Unc119 is upregulated during Th2 development. Unc119 expression is also augmented by prolonged TCR stimulation. Other reports show that prolonged TCR stimulation is required for Th2 cytokine production (26). Our results suggest that increased expression of Unc119 might facilitate the Th2 differentiation and block the Th1 development. Overexpression of Unc119 augments the amount of Lck at the plasma membrane and increases Lck and Itk enzymatic activities (this study, 14, 15). Unc119 overexpression inhibits Abl activation (17). Thus, Unc119 overexpression leads to the enhancement and inhibition of the Th2- and Th1-promoting signaling pathways, respectively. Interestingly, Th2 cells have been shown to have increased Lck activity (44).

The heightened expression of Unc119 protein in CD4 T cells from asthmatic subjects could result from a genetic predisposition (e.g., single nucleotide polymorphism) and/or exposure to the inflammatory milieu. Allergen may not be the only stimulus. The observed 66.3% increase in Unc119 expression in CD4 T cell population is unlikely to have its roots solely in allergen-specific T cells, which are rare and account for 0.0001–0.1% of the total T cell population (45). Thus, other stimuli (e.g., cytokines) are likely to affect the Unc119 level. We believe that heightened expression of Unc119 in T cells from asthmatic subjects results in skewed tyrosine kinase signaling. Increased concentration of Unc119 results in augmented Lck and Itk activities and in diminished Abl activity. These signaling events promote Th2 differentiation and function as well as airway inflammation. Interestingly, Unc119 expression is normal in allergic subjects without asthma. We speculate that the stimulation threshold for Unc119 induction is not reached in atopic subjects because of limited inflammation. The difference in Unc119 level between allergic and allergic asthmatic subjects could also be due to a genetic predisposition.

We have demonstrated an increased expression of Unc119 in the lung tissue from a mouse model of asthma. T cells are probably not the only cells that upregulate Unc119 expression under inflammatory conditions. Unc119 regulates other inflammatory cells: eosinophils and myofibroblasts (11, 16). We have previously reported that an elevation of the Unc119 protein level via protein transduction protects human eosinophils from apoptosis (11). Importantly, Unc119-transduced eosinophils survive in the absence of IL-5 and other survival-promoting cytokines. The inhibition of Unc119 function via transduction of a neutralizing Ab results in eosinophil apoptosis. Overexpression of Unc119 in human lung fibroblasts promotes their transformation into myofibroblasts (16). Conversely, Unc119 knockdown inhibits myofibroblast transformation. Interestingly, the Unc119 protein level is significantly higher in fibroblasts obtained from the normal human lung than in those from the skin (16). Thus, the lung milieu might promote increased Unc119 expression, thereby facilitating Th2 inflammation. We speculate that heightened Unc119 expression in multiple cell types is important for asthma pathogenesis.

Disclosures

The authors have no financial conflicts of interest.

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