

Th17-associated cytokines promote human airway smooth muscle cell proliferation

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ABSTRACT Increased airway smooth muscle (ASM) mass is a hallmark of airway remodeling in severe asthma. Th17-associated cytokines, particularly IL-17A, IL-17F, and IL-22, have been postulated to play a role in the pathogenesis of asthma. To investigate the *in vitro* effect of Th17 cytokines on the proliferation and survival of airway smooth muscle cells (ASMCs), human ASMCs from asthmatic and nonasthmatic subjects were incubated with IL-17A, IL-17F, or IL-22. The aforementioned cytokines demonstrated an ability to promote proliferation and survival of ASMCs from asthmatic and nonasthmatic subjects, which were mediated by selective activation of their corresponding receptors on ASMCs, including IL-17RA, IL-17RC, or IL-22R1, respectively. IL-17A and IL-17F-induced proliferation of ASMCs was dependent on ERK1/2 MAPK pathway, while IL-22-induced proliferation involved both ERK1/2 MAPK and NF- κ B pathways. The involvement of signaling pathways was further confirmed by the inhibition of proliferation by knockdown of ERK1/2 MAPK or NF- κ B p65 expression with pathway-specific siRNA. Together, our results show that Th17-associated cytokines promote proliferation and reduce the apoptotic rate of human ASMCs, raising the possibility that Th17 cytokines may contribute to increasing airway smooth muscle mass and airway remodeling in asthma.—Chang, Y., Al-Alwan, L., Risse, P.-A., Halayko, A. J., Martin, J. G., Baglole, C. J., Eidelman, D. H., Hamid, Q. Th17-associated cytokines promote human airway smooth muscle cell proliferation. *FASEB J.* 26, 000–000 (2012). www.fasebj.org

Key Words: asthma • apoptosis • ERK • NF- κ B

ASTHMA IS ASSOCIATED WITH changes in the structure of the airway wall, termed airway remodeling, a phenomenon that becomes prominent as disease severity

increases (1). Reports indicate that among the histopathological changes of airway remodeling, increased airway smooth muscle (ASM) mass plays a critical role in asthma pathogenesis (2, 3) and is believed to arise due to airway smooth muscle cell (ASMC) hyperplasia, hypertrophy or ASMC migration (3). Since culture of ASMCs from airway-obtained tissue has become possible, there has been considerable focus on the inflammatory mediators leading to proliferation of ASMCs, including TGF- β 1 (4), eotaxin, RANTES, IL-8, and MIP-1 α (5). Very recently, an *in vivo* study showed that overexpression of PDGF-BB could induce ASM hyperplasia in a mouse model of adenovirus overexpression and chronic allergen exposure (6).

Th17 cells, a proinflammatory CD4⁺ T-effector cell type, are characterized by production of IL-17A, IL-17F, and IL-22 (7). These cells have an important role in host defense against specific pathogens and are potent inducers of autoimmunity and tissue inflammation (8). Emerging studies suggest that Th17 cells and Th17-associated cytokines are involved in asthma, particularly in the severe phenotype (9–11). Recently, IL-17A was shown to mediate severe airway hyperresponsiveness in mice (12). Th17 cells and IL-17 may drive airway remodeling through the accumulation of neutrophils or by stimulating the release of matrix metalloproteases. IL-17 was also shown to increase the release of cytokines and growth factors from resident cells, including IL-6, IL-8, IL-11, GM-CSF, and VEGF (13–16). Previously, we investigated the role of Th17-associated cytokines, including IL-17A, IL-17F, and IL-22, on ASMC migration, and demonstrated the capacity of these cytokines to induce ASMC migration through differential signaling pathways (17). Our previous study has provided a basis for a role that Th17 cytokines may

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Abbreviations: ASM, airway smooth muscle; ASMC, airway smooth muscle cell; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor κ B; PI, propidium iodide; siRNA, short-interference RNA

play in airway remodeling in asthma through promoting ASMC migration. However, whether Th17 cytokines have a role in ASMC proliferation and survival is not known.

In the present study, we investigated the proliferation of ASMCs from both asthmatic and nonasthmatic subjects in response to Th17-associated cytokines IL-17A, IL-17F, and IL-22. Our findings suggest that Th17 cytokines play an important role increasing ASM mass and airway remodeling in asthma.

MATERIALS AND METHODS

Isolation and culture of human ASMCs

Primary cultures of human ASMCs were prepared from transplant specimens as described previously (18). Informed consent for ASMC harvesting was obtained from all subjects. ASMCs from asthmatic subjects were purchased from Lonza (Basel, Switzerland). ASMCs from nonasthmatic subjects were also purchased from Lonza and used as a control to evaluate the responses between different sources of cells. Cells were cultured in DMEM/F12 (1:1; Gibco; Invitrogen, Grand Island, NY, USA) supplemented with 10% FBS and 100 U/ml penicillin/streptomycin (Clonetics, Walkersville, MD, USA) at 37°C with 5% CO₂. Cells from the third to seventh passages were used and were identified as smooth muscle cells by positive immunohistochemical staining for smooth muscle-specific α -actin and positive identification of smooth muscle myosin heavy chain and calponin by Western blot analysis.

Proliferation assays

ASMCs were plated in 24-well plates and grown to 60–70% confluence in medium containing 5% FBS, after which they were starved for 48 h in 0.5% FBS-containing medium. Cells were then treated for 24, 48, or 72 h with different concentrations of IL-17A, IL-17F, or IL-22 (0, 0.1, 1, 10, and 100 ng/ml) or positive control 10 ng/ml of PDGF-BB (R&D Systems, Minneapolis, MN, USA), respectively. At 18 h before harvesting for proliferation assays, cells were pulsed with 3 μ Ci of ³H-thymidine/well. A Skatron Micro96 Cell Harvester (Skatron, Tranby, Norway) was used to isolate DNA following the manufacturer's instructions, and then radioactivity was measured with a scintillation counter (PerkinElmer, Waltham, MA, USA). In some experiments, the proliferation of cells was evaluated by BrdU incorporation (BrdU FITC kit; BD Biosciences, Franklin Lakes, NJ, USA).

To ensure cytokine specificity, we pretreated cytokines for 1 h with mouse monoclonal neutralizing antibodies or the isotype controls prior to the stimulation of cells. Here, IL-17A was inhibited by the addition of 10 μ g/ml mouse anti-human IL-17A mAb (R&D Systems), IL-17F by 10 μ g/ml mouse anti-human IL-17F mAb (R&D Systems), and IL-22 by 10 μ g/ml mouse anti-human IL-22 mAb (R&D Systems). In each case, results were compared to the effect of adding the corresponding isotype controls.

To confirm that the Th17 cytokine-induced proliferation was dependent on receptor activation, we pretreated ASMCs with anti-IL-17RA mAb and/or anti-IL-17RC Ab or anti-IL-22R mAb (R&D Systems). Results were compared to the corresponding isotype controls.

We used pharmacological inhibitors against various pathways known to potentially contribute to increased ASMC proliferation. Specifically, we used the p38 MAPK inhibitor

BIRB796 (0.1 μ M; Axon Medchem BV, Groningen, The Netherlands; ref. 19), the extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein kinase (MAPK) inhibitor PD184352 (2 μ M; US Biological, Swampscott, MA, USA; ref. 20), and the nuclear factor κ B (NF- κ B) inhibitor helenalin (1 μ M; Enzo Life Sciences, Ann Arbor, MI, USA; ref. 21). In each case, ASMCs were incubated with the inhibitor for 1 h at 37°C prior to the stimulations. All results were compared to the corresponding vehicle control (DMSO).

Immunohistochemistry

Biopsies from the subsegmental bronchi were obtained from one patient with clinically diagnosed severe asthma or a control subject at the Montreal Chest Institute (McGill University Health Centre). Cryostat sections (5 μ m thick) were fixed in 4% paraformaldehyde for 6 min. After washing in PBS containing 0.1% Tween 20, sections were incubated with 6% hydrogen peroxide for 30 min at room temperature to block endogenous peroxidase activity. The slides were pretreated with universal blocking solution (Dako, Carpinteria, CA, USA) and incubated overnight at 4°C using diluted rabbit anti-human α -SMA pAb (1:1000, ab32575; Abcam, Cambridge, MA, USA), mouse anti-human IL-17RA mAb (1:100, MAB177; R&D Systems), goat anti-human IL-17RC pAb (1:200, AF2269; R&D Systems) or goat anti-human IL-22R1 pAb (1:200, AF2770, R&D Systems). Nonimmune isotypes were used as a negative control. The slides were then incubated with a biotinylated secondary antibody for 30 min at room temperature, followed by streptavidin-horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA) for 30 min and visualized with DAB/hydrogen peroxide (DAB Kit; Dako). The sections were counterstained with hematoxylin, dehydrated, cleared, and coverslipped.

Apoptosis assays

ASMCs were cultured in 12-well plates and grown to 60–70% confluence in medium containing 5% FBS. Cells were then starved for 48 h in 0.5% FBS-containing medium and treated with medium alone, IL-17A/F, or IL-22 for 24 h (cells from nonasthmatic subjects) or 48 h (cells from asthmatic subjects). Then annexin V and propidium iodide (PI) staining (annexin V-FITC apoptosis detection kit, BD Biosciences) was used to quantify the percentage of cells undergoing early apoptosis. The cells were analyzed on a flow cytometer (BD FACSCalibur; BD Biosciences).

ERK1/2- and NF- κ B p65-knockdown by short-interference RNA (siRNA) transfection

ASMCs were plated in 24-well plates and grown to 60–70% confluence in medium containing 5% FBS. The cells were then transfected with siRNA targeting ERK1/2 (Cell Signaling, Danvers, MA, USA) or NF- κ B p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using the siRNA Reagent System (Santa Cruz Biotechnology). The scramble siRNA (Santa Cruz Biotechnology) was used as a negative control. After 48 h starvation in 0.5% FBS, ASMCs were stimulated with Th17 cytokines (48 h for ASMCs from nonasthmatic subjects and 72 h for ASMCs from asthmatic subjects) and prepared for the proliferation assay, as described previously. The efficiency of knockdown was examined by Western blotting for total ERK1/2 or NF- κ B p65 in nonstimulated cells. GAPDH (Millipore, Billerica, MA, USA) or β -actin (Abcam) were used as loading controls.

Assessment of ERK1/2 MAPK and p65 NF- κ B phosphorylation

Nonconfluent ASMCs were incubated in starvation medium with 0.5% FBS for 48 h. Cells were then stimulated with 0.1 ng/ml IL-17A, 1 ng/ml IL-17F, or 0.1 ng/ml IL-22 for nonasthmatic subjects or 100 ng/ml IL-17A, 1 ng/ml IL-17F, or 100 ng/ml IL-22 for asthmatic subjects for 0, 5, 15, 30, 45, and 60 min. Protein was extracted and loaded (10 μ g) on a 10% acrylamide SDS-PAGE next gel (Amresco, Solon, OH, USA) for protein separation, followed by transfer to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The blots were then blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 1 h at room temperature and then incubated overnight at 4°C with antibodies specific for total ERK1/2 MAPK (Cell Signaling), phosphorylated ERK1/2 MAPK (Cell Signaling), phosphorylated NF- κ B p65 (GenScript, Piscataway, NJ, USA), or GAPDH (Millipore). After washing with 0.1% Tween 20 in PBS, the membranes were incubated with a 1:15,000 dilution of IRDye 680 goat anti-mouse IgG and IRDye 680 goat anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA, USA) in blocking buffer. Membranes were scanned and analyzed using an Odyssey IR scanner using Odyssey 3.0 imaging software (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

All data are presented as means $\pm$ SE. Statistical analysis for cytokine-induced proliferation was performed by ANOVA followed by the *post hoc* Bonferroni test. The paired *t* test was performed for other assays. Values of $P < 0.05$ were regarded as statistically significant. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA).

RESULTS

Th17 cytokines promote proliferation of ASMCs from both asthmatic and nonasthmatic subjects

To investigate the effect of Th17 cytokines on proliferation of ASMCs, cells from both nonasthmatic and asth-

matic subjects were treated with different concentrations of IL-17A/F or IL-22 for 24, 48, and 72 h (Fig. 1). For the cells from nonasthmatic subjects, a significant increase in ^{3} H-thymidine incorporation was detected after incubating ASMCs with Th17 cytokines for 48 h compared to the medium (0.1 ng/ml of IL-17A: $154.7 \pm 12.8\%$, $P < 0.05$; 1 ng/ml of IL-17F: $150.6 \pm 22.5\%$, $P < 0.05$; 0.1 ng/ml of IL-22: $142 \pm 14.2\%$, $P < 0.05$; Fig. 1A). A similar proliferative response to Th17 cytokines was observed in the cells purchased from Lonza compared with the cells isolated from transplant specimens (Supplemental Fig. S1A). A different trend was observed with ASMCs from subjects with asthma, where the increase in proliferation of ASMCs was found only at 72 h, and higher concentrations of IL-17A and IL-22 were required for this effect (100 ng/ml of IL-17A: $156.1 \pm 7.4\%$, $P < 0.001$; 1 ng/ml of IL-17F: $171.5 \pm 20.8\%$, $P < 0.001$; 100 ng/ml of IL-22: $153.7 \pm 26.3\%$, $P < 0.05$; Fig. 1B). On both types of ASMCs, we found no evidence of synergistic interaction when the cytokines were administered in combination (IL-17A/F or IL-17A/F combined with IL-22; data not shown). An increase of 2- or 3-fold in proliferation was seen after treatment of asthmatic and nonasthmatic subject ASMCs with PDGF-BB as a positive control (22). Comparable results were observed by BrdU incorporation (Supplemental Fig. S1B).

Pretreatment of cytokines with neutralizing antibodies but not their isotype controls inhibited Th17 cytokine-induced proliferation of ASMCs (Fig. 2). Taken together, our results suggest that Th17 cytokines stimulate proliferation of ASMCs from both asthmatic and nonasthmatic subjects.

Th17 cytokines reduce ASMC apoptosis

Because an increase in ASM mass could be due to excessive cell proliferation or decreased apoptosis *in vivo*, we examined the effect of Th17 cytokines on ASMC

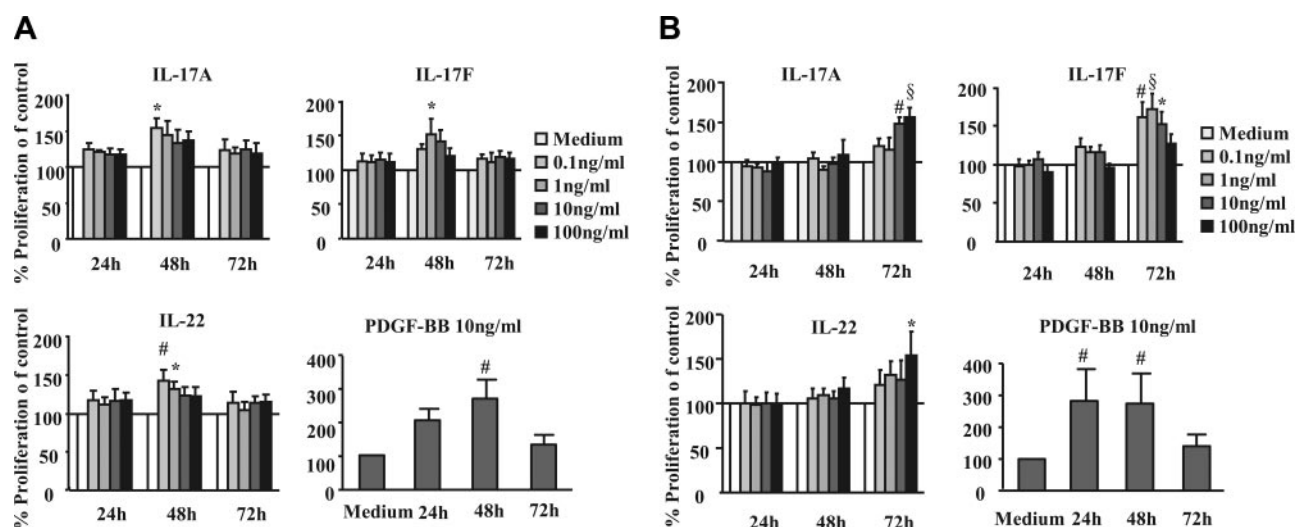


Figure 1. Effect of Th17 cytokines on ASMC proliferation. Nonconfluent ASMCs from nonasthmatic subjects (A) and asthmatic subjects (B) were treated with 0.1–100 ng/ml of IL-17A, IL-17F, or IL-22 for 24, 48, and 72 h; cell proliferation was assayed by ^{3} H-thymidine incorporation. PDGF-BB was used as a positive control. $n = 7$ for nonasthmatic subjects; $n = 5$ for asthmatic subjects. * $P < 0.05$, # $P < 0.01$, § $P < 0.001$ vs. medium control.

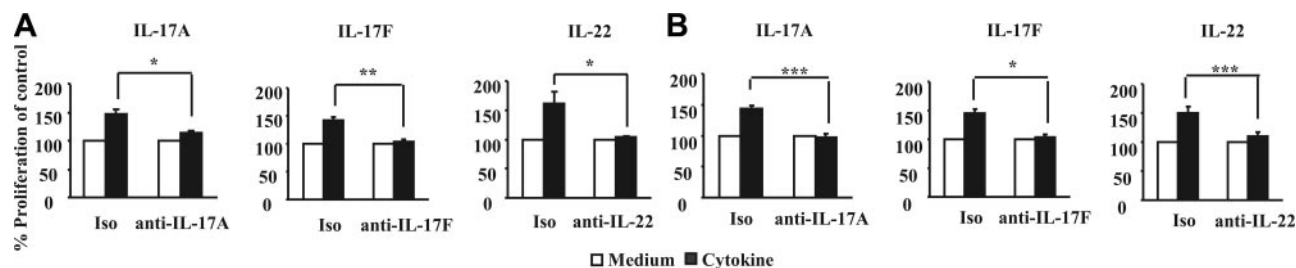


Figure 2. Cytokine specificity of Th17 cytokine-promoted proliferation of ASMCs. ASMCs from nonasthmatic subjects (A) and asthmatic subjects (B) were pretreated with neutralizing antibodies against Th17 cytokines or isotype control antibodies prior to the stimulation with cytokines. Cells were treated with 0.1–100 ng/ml of IL-17A, IL-17F, or IL-22 for 24, 48, and 72 h. Cell proliferation was assayed by ^3H -thymidine incorporation. $n = 5/\text{group}$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

apoptosis. For this experiment, we chose concentrations that induced the largest increase in proliferation (Fig. 1). For the cells from nonasthmatic subjects, all the cytokines significantly reduced apoptosis (annexin V⁺PI[−]; 0.1 ng/ml IL-17A: $47.5 \pm 15.7\%$; 1 ng/ml IL-17F: $64.6 \pm 11.9\%$; 0.1 ng/ml IL-22: $65.2 \pm 20.7\%$; $P < 0.05$) as compared to negative control (Fig. 3). Similar effect was seen in cells from asthmatic subjects (100 ng/ml IL-17A: $62.7 \pm 3.4\%$; 1 ng/ml IL-17F: $80.0 \pm 10.0\%$; 100 ng/ml IL-22: $58.5 \pm 9.5\%$; $P < 0.05$; Fig. 3). These results suggest that Th17 cytokines have an antiapoptotic effect on ASMCs, resulting in enhanced survival and increased ASM mass.

Th17 cytokine-induced ASMC proliferation is dependent on Th17 cytokine receptors

We have shown previously that ASMCs express Th17 cytokine receptors, namely IL-17RA and IL-17RC, both of which are receptors for IL-17A and IL-17F. We also demonstrated the expression of IL-22R1, a subunit of the IL-22 receptor complex, on ASMCs (23). We have performed immunohistochemistry staining for IL-17RA, IL-17RC, and IL-22R1 on human bronchial

airway biopsies from both nonasthmatic subjects and subjects with severe asthma. The expression of these receptors was observed on the ASM area (Supplemental Fig. S2). To further investigate the importance of these receptors in modulating the proliferative effect of Th17 cytokines, we attempted to selectively block these receptors by using neutralizing antibodies. For ASMCs from both nonasthmatic and asthmatic subjects (Fig. 4), blockade of IL-17RA, IL-17RC, or both significantly reduced IL-17A- or IL-17F-induced proliferation. For IL-22-induced proliferation of ASMCs, blockade of IL-22R1 significantly inhibited the proliferation of cells from both nonasthmatic and asthmatic subjects ($P < 0.05$ for nonasthmatic subject ASMCs, $P < 0.01$ for asthmatic subject ASMCs; Fig. 4). This suggests that Th17 cytokine-induced ASMC proliferation is mediated by selective activation of receptors on ASMCs.

ERK1/2 MAPK and NF- κ B pathways are involved in Th17 cytokine-induced proliferation

To explore the signaling pathways involved in Th17 cytokine-induced proliferation of ASMCs, we used

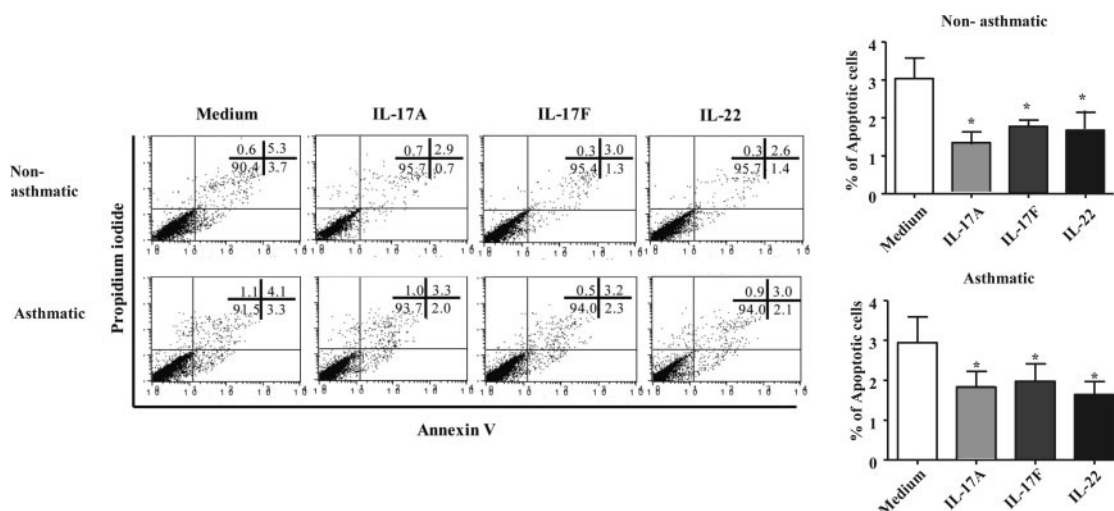


Figure 3. Effect of Th17 cytokines on apoptosis of ASMC. ASMCs from nonasthmatic subjects were treated with 0.1 ng/ml of IL-17A, 1 ng/ml of IL-17F, or 0.1 ng/ml of IL-22 for 24 h. ASMCs from asthmatic subjects were treated with 100 ng/ml of IL-17A, 1 ng/ml of IL-17F, or 100 ng/ml of IL-22 for 48 h. Cells were stained with annexin V and PI. Annexin V⁺PI[−] stained cells represent the early apoptotic cells. $n = 5$ for nonasthmatic subjects; $n = 4$ for asthmatic subjects. * $P < 0.05$ vs. medium control.

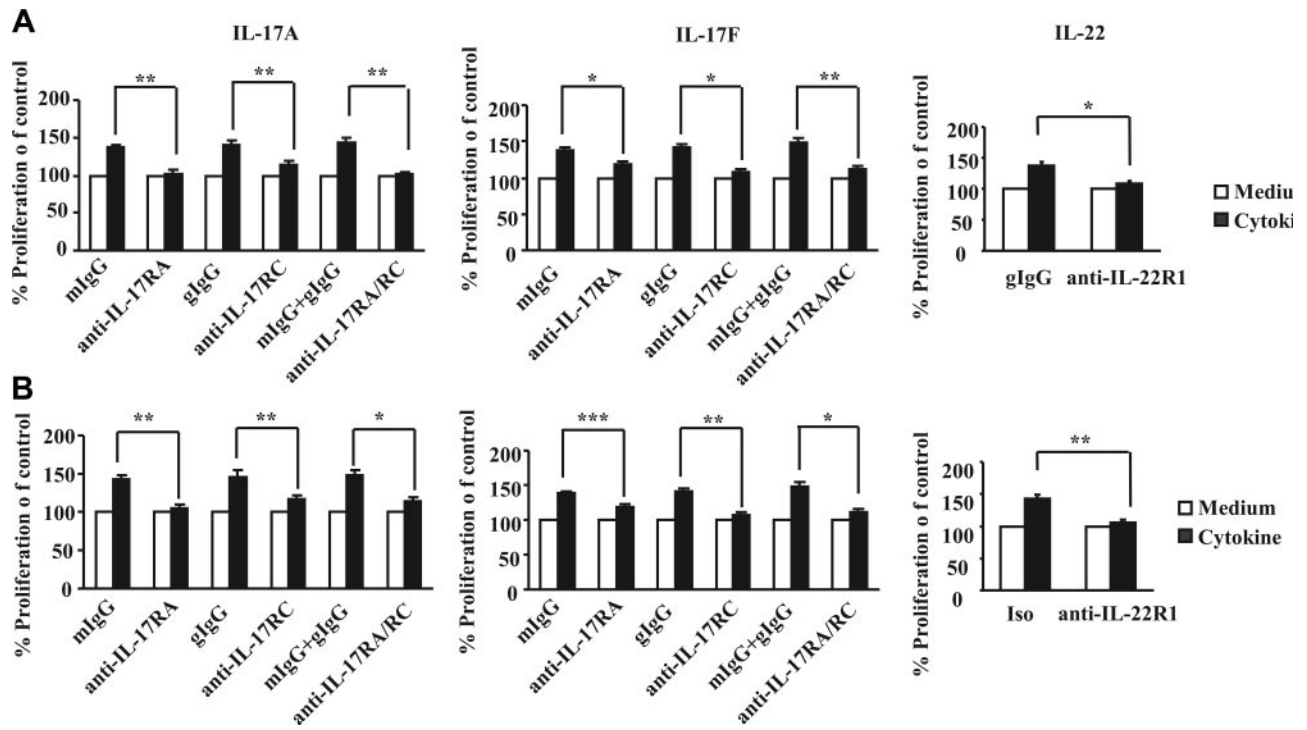


Figure 4. Involvement of Th17 cytokine receptors in the ASMC proliferation induced by Th17 cytokines. ASMCs from nonasthmatic subjects (A) and asthmatic subjects (B) were pretreated with neutralizing antibodies against IL-17RA, IL-17RC, or IL-22R1 prior to the stimulations. ASMCs from nonasthmatic subjects were treated with 0.1 ng/ml of IL-17A, 1 ng/ml of IL-17F, or 0.1 ng/ml of IL-22 for 48 h. ASMCs from asthmatic subjects were treated with 100 ng/ml of IL-17A, 1 ng/ml of IL-17F or 100 ng/ml of IL-22 for 72 h. Cell proliferation was assayed by ^3H -thymidine incorporation. $n = 5/\text{group}$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

pharmacologic inhibitors against p38 MAPK, ERK1/2 MAPK, and NF- κ B pathways, as these pathways are known to be activated by Th17 cytokines (17, 24, 25).

For cells from both nonasthmatic (Fig. 5A) and asthmatic subjects (Fig. 5B), PD184352 (2 μM), an inhibitor of ERK1/2 MAPK (26), significantly suppressed ASMC

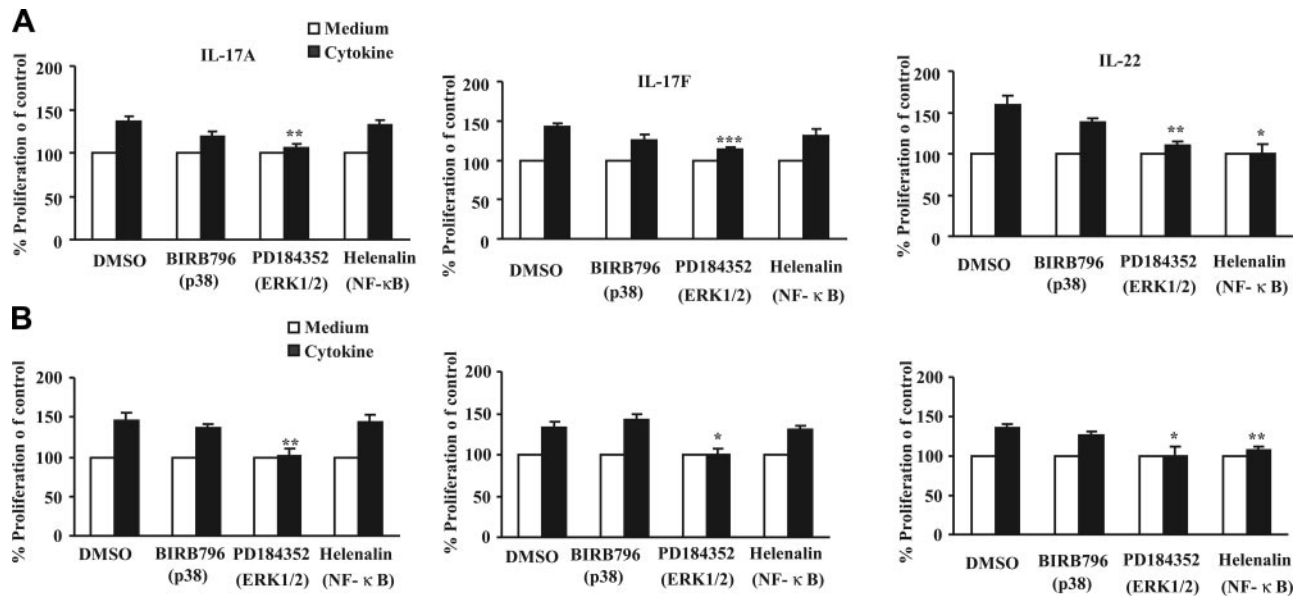


Figure 5. Effect of pharmacologic inhibitors for signaling pathways on Th17 cytokine-induced proliferation of ASMCs. ASMCs from nonasthmatic subjects (A) and asthmatic subjects (B) were pretreated with p38 MAPK inhibitor BIRB796 (0.1 μM), ERK1/2 MAPK inhibitor PD184352 (2 μM), or NF- κ B inhibitor helenalin (1 μM) prior to the stimulations. ASMCs from nonasthmatic subjects were treated with 0.1 ng/ml of IL-17A, 1 ng/ml of IL-17F, or 0.1 ng/ml of IL-22 for 48 h. ASMCs from asthmatic subjects were treated with 100 ng/ml of IL-17A, 1 ng/ml of IL-17F, or 100 ng/ml of IL-22 for 72 h. Cell proliferation was assayed by ^3H -thymidine incorporation. $n = 5/\text{group}$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

proliferation induced by IL-17A (nonasthmatic subjects: $69.2 \pm 12.9\%$, $P < 0.05$; asthmatic subjects: $76.4 \pm 10.8\%$, $P < 0.01$), IL-17F (nonasthmatic subjects: $71.0 \pm 3.3\%$, $P < 0.01$; asthmatic subjects: $73.2 \pm 12.9\%$, $P < 0.05$) and IL-22 (nonasthmatic subjects: $93.1 \pm 13.3\%$, $P < 0.01$; asthmatic subjects: $70.1 \pm 17.7\%$, $P < 0.05$). Administration of p38 MAPK and NF- κ B inhibitors had no effect on IL-17A- and IL-17F-induced ASMC proliferation. In contrast, helenalin (an inhibitor for NF- κ B, 1 μ M) had a significant inhibitory effect on IL-22-induced ASMC proliferation (nonasthmatic subjects: $71.4 \pm 24.5\%$, $P < 0.05$; asthmatic subjects: $67.7 \pm 17.6\%$, $P < 0.05$).

Knockdown of ERK1/2 and NF- κ B p65 inhibited the Th17 cytokine-induced proliferation

To further confirm the involvement of ERK1/2 and NF- κ B pathways in Th17 cytokine-induced ASMC pro-

liferation, we used siRNA to knock down the expression of ERK1/2 or NF- κ B p65; the efficiency of knockdown was assessed by Western blot (51.9 ± 3.5 and $53.9 \pm 6.9\%$ of ERK1/2 and NF- κ B p65 proteins, respectively; **Fig. 6A**). After siRNA transfection, ASMCs from both asthmatic and nonasthmatic subjects showed partial reduction in their proliferation on stimulation with Th17 cytokines (**Fig. 6B**).

ERK1/2 MAPK and NF- κ B pathways are activated by Th17 cytokines on ASMCs

We sought direct evidence of ERK1/2 MAPK phosphorylation following administration of exogenous IL-17A, IL-17F, and IL-22. As shown in **Fig. 7**, stimulation by IL-17A and IL-17F resulted in significant phosphorylation of ERK1/2 MAPK on ASMCs from nonasthmatic subjects, starting at 5 min up to 45 min, and from 5 to

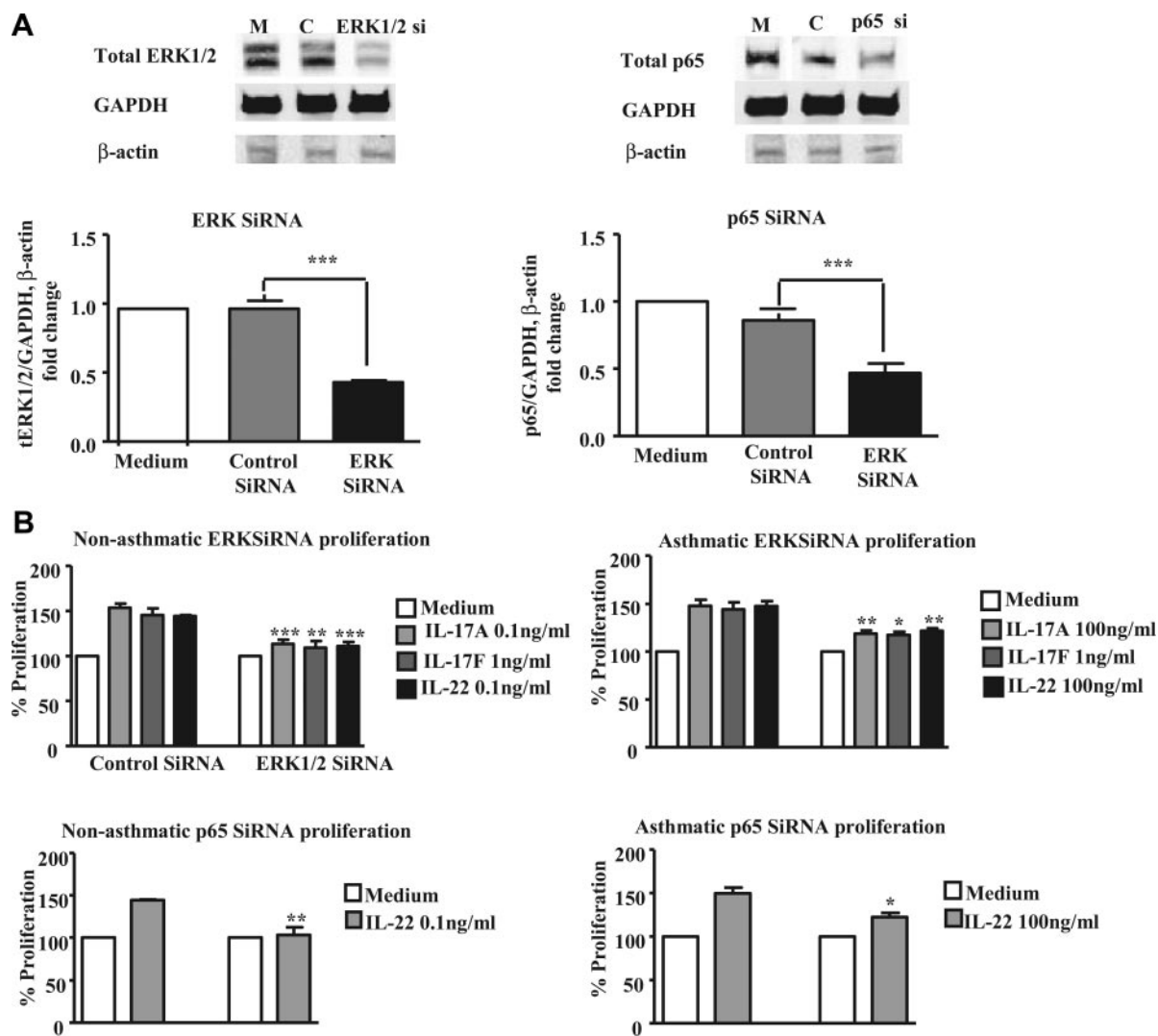


Figure 6. Effect of ERK1/2 and NF- κ B p65 knockdown on the Th17 cytokine-induced ASMC proliferation. **A**) Efficiency of ERK1/2 and NF- κ B knockdown by siRNA transfection. Western blot analysis was performed for ERK1/2 and p65 immunostaining. The specific protein was normalized with GAPDH intensity. $n = 3$ subjects. M, medium; C, control siRNA. $***P < 0.001$. **B**) Proliferation assay of ASMCs transfected with control siRNA or specific siRNA targeting on ERK1/2 or NF- κ B p65. Incubation time with Th17 cytokines was 48 h for ASMCs from nonasthmatic subjects and 72 h for ASMCs from asthmatic subjects. $n = 3$ /group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. medium control.

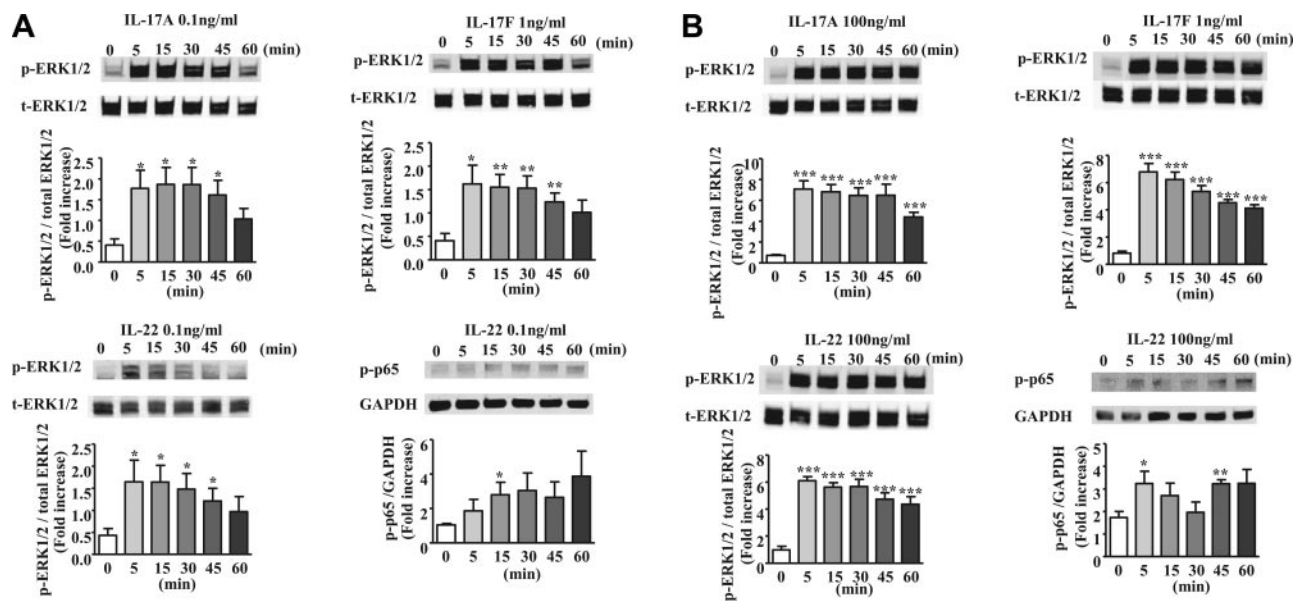


Figure 7. Activation of ERK1/2 MAPK and NF- κ B pathways on ASMCs induced by Th17 cytokines. Nonconfluent and growth-arrested ASMCs from nonasthmatic subjects (A) and asthmatic subjects (B) were stimulated with IL-17A, IL-17F, IL-22, or medium for 5, 15, 30, 45, and 60 min. Images are representative of similar results from 5 independent experiments. Phosphorylation of ERK1/2 MAPK and p65 NF- κ B was analyzed by calculating the ratio with GAPDH. $n = 5/\text{group}$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control.

60 min on ASMCs from asthmatic subjects, after stimulation with IL-17A and IL-17F. We also examined the effect of IL-22 on ERK1/2 MAPK and NF- κ B phosphorylation at a concentration of 0.1 ng/ml for cells from nonasthmatic subjects and 100 ng/ml for cells from asthmatic subjects. Interestingly, IL-22 treatment of ASMCs from nonasthmatic subjects induced a transient phosphorylation of ERK1/2 MAPK and NF- κ B (Fig. 7A), while their phosphorylation was more prolonged in IL-22-treated cells from asthmatic subjects (Fig. 7B). Collectively, our results highlight the signaling pathways involved in Th17 cytokine-induced proliferation of ASMCs, where all these cytokines are dependent on the ERK1/2 MAPK pathway, and IL-22 is also dependent on the NF- κ B pathway.

DISCUSSION

Although the relationship between ASM and asthma symptoms remains elusive, it is reasonable to postulate that increased ASM mass could contribute to increased airway narrowing and airflow obstruction (27). As the importance of the Th-17 family of cytokines in asthma emerges, we sought to explore the relationship of those cytokines to the enhancement of ASMC proliferation and survival, which have the potential to contribute to increased ASM mass. In the present study, we demonstrated for the first time that Th17 cytokines promote proliferation of ASMCs from both nonasthmatic and asthmatic subjects and enhance cell survival by reducing apoptosis. IL-17A and IL-17F, as well as IL-22 led to enhanced proliferation of ASMCs in a selective, receptor-mediated fashion. Induced proliferation of normal

control ASMCs required lower concentrations of Th17 cytokines and was achieved within 48 h of stimulation, compared to proliferation of ASMCs from subjects with asthma, which required higher concentrations and longer stimulation time (72 h; Fig. 1). This suggests that in subjects with asthma, ASMCs may be less sensitive in their response to Th17 cytokines. This phenomenon is not due to the difference in expression of Th17 cytokine receptors on ASMCs, as demonstrated by flow cytometry (data not shown). However, switching between contractile and proliferative phenotypes occurs in cultured ASMCs (28). Very recently, Kudo *et al.* (29), using mouse tracheal rings and human tissue, found that 100 ng/ml of IL-17A enhanced ASM contraction. Thus, cultured cells may require concentrations of <100 ng/ml of IL-17A to induce switching from contractile to proliferative phenotypes.

In addition to cell proliferation, the excess of ASM in asthma may be explained by increased survival of ASM cells due to lower rate of apoptosis. IL-17 was previously shown to directly induce apoptosis of neutrophils (30) or indirectly by decreasing GM-CSF-mediated survival (31). More recently, IL-22 was shown to protect airway epithelial cells from bleomycin-induced apoptosis, and this protection was reversed after coadministration of IL-17A (32). Conversely, we show that besides IL-22, both IL-17A and IL-17F reduce the apoptosis rate of ASMCs from subjects with or without asthma (Fig. 3). Interestingly, there was a significant proportional correlation between Th17-induced proliferation and decreased apoptosis of ASMCs (data not shown), indicating a close association between the two mechanisms as a result of Th17 cytokine stimulation.

Both IL-17A and IL-17F have been shown to bind to

IL-17RA and IL-17RC. Therein, we used neutralizing antibodies to confirm the importance of receptor binding in the previous phenomena. As shown in Fig. 4, all receptors contributed to the increase in cell proliferation. Blockade of either IL-17RA or IL-17RC partially prevented the increase in proliferation induced by exogenous administration of IL-17A or IL-17F. Whereas IL-17A and IL-17F bind IL-17RC with similar affinities, the affinity of IL-17F for IL-17RA is considerably lower (33). However, it has been argued that the binding of the IL-17RC receptor may not be essential for the activity of IL-17A and IL-17F (34–36). To the contrary, our results support an important role of IL-17RC for the function both IL-17A and IL-17F in human ASMCs.

The ERKs MAPK [p42/p44 (ERK1/2)] pathway has been associated with growth factor-induced ASMC proliferation (37). Furthermore, the activation of MAPK pathways on ASMCs in response to IL-17 has been established (9, 17). To explore the effect of the ERK1/2 signaling pathway in the enhanced proliferation of ASMCs in response to Th17 cytokines, we administered PD184352, which blocks the phosphorylation of ERK and thus ERK pathway activity (26). We found that PD184352 significantly inhibited IL-17A-, IL-17F-, and IL-22-induced proliferation (Fig. 5). We further confirmed the role of ERK1/2 in Th17 cytokine-induced proliferation by knocking down ERK1/2 expression using siRNA transfection (Fig. 6). The activation of ERK1/2 MAPK by Th17 cytokines was confirmed by the assessment of ERK1/2 MAPK phosphorylation (Fig. 7). Interestingly, higher levels of phosphorylated ERK1/2 were observed in ASMCs from asthmatic subjects (~6-fold changes) compared to that from nonasthmatic subjects (~2-fold changes). Regardless, the proliferation observed in ASMCs from asthmatic subjects was not different from cells from nonasthmatic subjects in response to Th17 cytokines. Thus, it is possible that the increase in ERK1/2 pathway activation due to Th17 cytokines treatment in ASMCs from asthmatic subjects may be displayed through different outcomes that are not studied here. Taken together, the difference in ERK1/2 phosphorylation raises the possibility that Th17 cytokines have differential effects on cells from asthmatic and nonasthmatic subjects.

The NF- κ B pathway has been shown to regulate NADPH oxidase-induced (38) and platelet-activating factor-induced (39) ASMC proliferation. In this study, IL-22-induced ASMC proliferation was also inhibited by the administration of helenalin, a specific NF- κ B inhibitor. Helenalin is known to modify the NF- κ B/I κ B complex, preventing the release of I κ B, and thus inhibiting NF- κ B pathway activation (40). These results were confirmed by siRNA knockdown of NF- κ B p65. However, only partial inhibition of proliferation induced by IL-22 was obtained after siRNA transfection, possibly due to the efficiency of NF- κ B p65 knockdown (53.9 $\pm$ 6.9%). We further reinforced our findings by exploring IL-22-induced NF- κ B p65 phosphorylation, which suggests that, in contrast to IL-17A and IL-17F,

the effect of IL-22 on ASMC proliferation is in part dependent on NF- κ B activity.

In summary, we have demonstrated that IL-17A, IL-17F, and IL-22 have the capacity to enhance ASMC proliferation. The effects of IL-17A, IL-17F, and IL-22 were receptor specific although they exerted their effects through the same signaling pathway, ERK1/2 MAPK. Of interest, however, we found that in addition to this pathway, IL-22-enhanced ASMC proliferation was also mediated through the NF- κ B pathway, suggesting that there are multiple mechanisms by which ASMC proliferation can be induced in the context of Th17 inflammation. This raises the possibility that Th17 cytokines may play a role in increasing ASM mass and airway remodeling in asthma and provides a clear evidence of a new and important mechanism that may be involved in promoting airway remodeling in this disease. **[FJ]**

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