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# Critical Role for STAT3 in IL-17A-Mediated CCL11 Expression in Human Airway Smooth Muscle Cells<sup>1</sup>

Ali Saleh,\* Lianyu Shan,\* Andrew J. Halayko,<sup>†</sup> Sam Kung,\* and Abdelilah S. Gounni<sup>2\*</sup>

IL-17A has been shown to be expressed at higher levels in respiratory secretions from asthmatics and to correlate with airway hyperresponsiveness. Although these studies raise the possibility that IL-17A may influence allergic disease, the mechanism remains unknown. We previously demonstrated that IL-17A mediates CC chemokine (CCL11) production from human airway smooth muscle (ASM) cells. In this study, we demonstrate that STAT3 activation is critical in IL-17A-mediated CCL11 expression in ASM cells. IL-17A mediated a rapid phosphorylation of STAT3 but not STAT6 or STAT5 in ASM cells. Interestingly, transient transfection with wild-type or mutated CCL11 promoter constructs showed that IL-17A-mediated CCL11 expression relies on the STAT6 binding site. However, STAT3 but not STAT6 in vivo binding to the CCL11 promoter was detected following IL-17A stimulation of ASM cells. Overexpression of DN STAT3 (STAT3 $\beta$ ) abolishes IL-17A-induced CCL11 promoter activity. This effect was not observed with STAT6 DN or the STAT3 mutant at Ser<sup>727</sup>. Interestingly, disruption of STAT3 activity with the SH2 domain binding peptide, but not with control peptide, results in a significant reduction of IL-17A-mediated STAT3 phosphorylation and CCL11 promoter activity. IL-17A-mediated CCL11 promoter activity and mRNA were significantly diminished in STAT3- but not STAT6-silenced ASM cells. Finally, IL-17A-induced STAT3 phosphorylation was sensitive to pharmacological inhibitors of JAK2 and ERK1/2. Taken together, our data provide the first evidence of IL-17A-mediated gene expression via STAT3 in ASM cells. Collectively, our results raise the possibility that the IL-17A/STAT3 signaling pathway may play a crucial role in airway inflammatory responses. *The Journal of Immunology*, 2009, 182: 3357–3365.

Human airway smooth muscle (ASM)<sup>3</sup> cells are key determinants of asthma owing to their ability to contract in response to inflammatory cell products (1). Due to their intrinsic phenotype plasticity, airway myocytes also exhibit a capacity for multifunctional behavior and are actively involved in local inflammation and fibrosis (2). Emerging evidence suggests that ASM cells can also directly contribute to the pathogenesis of asthma by altering the interstitial extracellular matrix, expressing cell adhesion and costimulatory molecules as well as secreting multiple proinflammatory cytokines and chemokines (3, 4). These responses can perpetuate airway inflammation and act on the hyperplastic and hypertrophic growth of ASM cells, contributing to airway hyperresponsiveness and the development of airway remodeling (5).

IL-17A is the founding member of an emerging cytokine family with pleiotropic biological activities and is produced by a distinct class of CD4<sup>+</sup> cells recently named Th17 cells (6). Exaggerated levels of IL-17A are observed in many chronic inflammatory disorders (7, 8), suggesting a potential role of this cytokine in the

initiation or maintenance of inflammatory responses. Although IL-17A has been shown to be present in higher levels in respiratory secretions from asthmatics (9) and increased concentrations of IL-17A correlate with airway hyperresponsiveness (10), the mechanism by which this cytokine may influence allergic disease processes remains unknown.

STAT3 (signal transducer and activator of transcription 3) is a cytoplasmic protein that functions as a transcriptional activator (11, 12). STAT3 protein exists in a latent form in the cytoplasm and become phosphorylated on tyrosine residues upon receptor activation by cytokines such as IL-6. This phosphorylation is obligatory for STAT3 activation and mediates dimerization via phospho-Tyr-SH2 domain interactions, resulting in translocation of the STAT3 dimers to the nucleus where they bind to sequence-specific DNA elements. Studies of isoform-specific transgenic knockout mouse models revealed that among all the STATs, STAT3 is the only isoform that leads to an embryonic lethal phenotype at days 6–7 of fetal development. Furthermore, none of the seven known STAT proteins can compensate its function loss (12).

Previously, we have demonstrated that IL-17A can induce CCL11 expression in human ASM cells (13). In the present study, we investigate the role of STAT3 in IL-17A-mediated CCL11 expression by ASM cells. Using different approaches, we demonstrated that the STAT3 pathway is required for IL-17A induction of CCL11 expression by ASM cells. Our data present the first evidence of IL-17A-mediated gene expression via STAT3 in ASM cells, thus highlighting the importance of this pathway in airway inflammation.

## Materials and Methods

### Reagents and Abs

Recombinant human IL-17A, IL-1 $\beta$ , and IL-4 were purchased from R&D Systems. Anti-phosphotyrosine STAT6 was purchased from BD Biosciences. Mouse mAb anti-phosphotyrosine STAT3 (Y705), affinity-purified rabbit anti-total STAT3, and STAT6 were from Santa Cruz Biotechnology. The p38 MAPK inhibitor (SB203580), the p42/p44 ERK inhibitor

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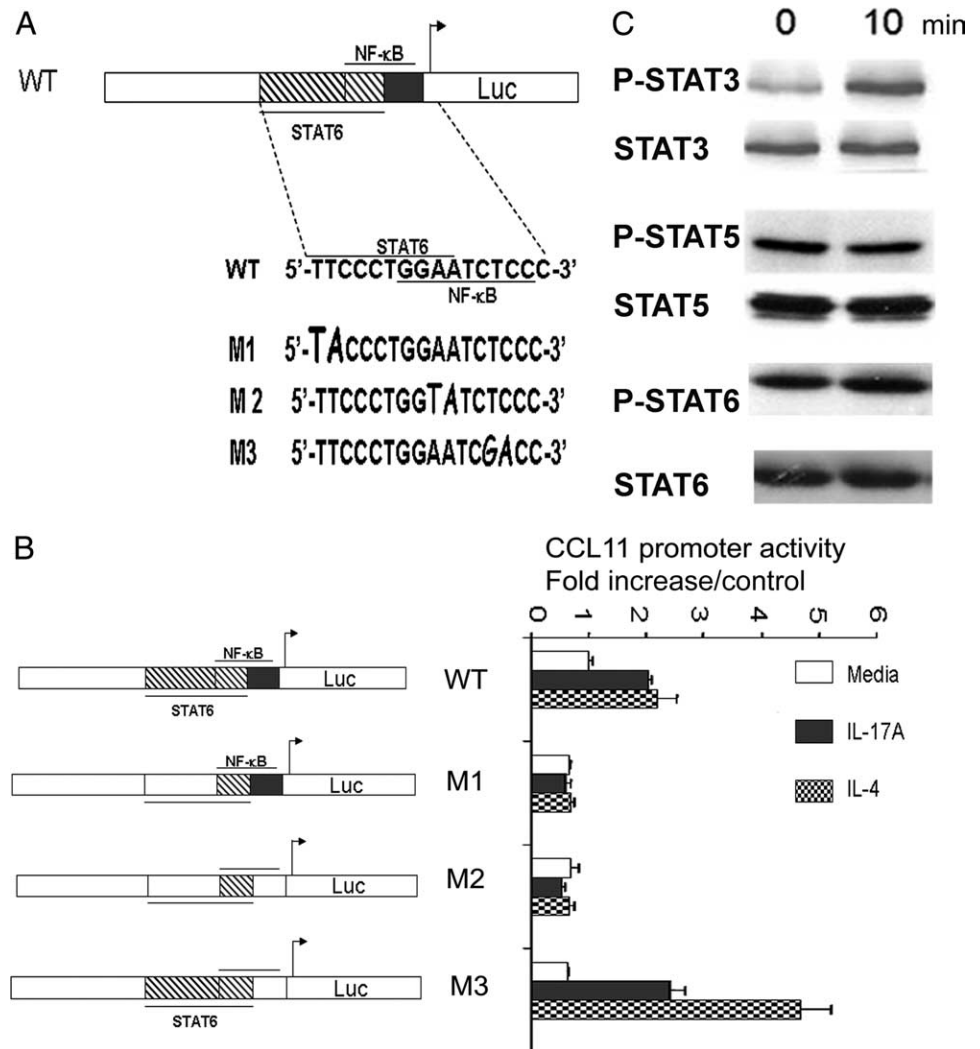
<sup>1</sup> This work was supported by Canadian Institutes of Health Research Grant MOP 53104 to (A.S.G.).

<sup>2</sup> Address correspondence and reprint requests to Dr. Abdelilah S. Gounni, Department of Immunology, Faculty of Medicine, University of Manitoba, 606 Basic Medical Sciences Building, 730 William Avenue, Winnipeg, R3E0W3 Manitoba, Canada. E-mail address: gounni@cc.umanitoba.ca

<sup>3</sup> Abbreviations used in this paper: ASM, airway smooth muscle; ChIP, chromatin immunoprecipitation; DN, dominant negative; RLU, relative luciferase unit; ShRNA, short hairpin RNA; tGFP, turboGFP; WT, wild type.

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**FIGURE 1.** IL-17A-mediated CCL11 promoter activity requires a STAT6 binding site. **A**, Schematic presentation depicting the sequences of the WT, STAT6 (M1), NF- $\kappa$ B (M3), and double mutant STAT6/NF- $\kappa$ B (M2) CCL11 promoter luciferase (Luc) constructs used. **B**, IL-17A inducibility of CCL11 promoter luciferase (Luc) reporter constructs in transiently transfected human ASM cells. ASM cells were transfected with a WT, STAT6 (M1), NF- $\kappa$ B (M3), or double mutant (M2) CCL11 promoter luciferase reporter plasmid. Twenty-four hours post-transfection, cells were incubated with or without IL-17 (10 ng/ml) or IL-4 (10 ng/ml) for 12 h and then harvested. The luciferase activity was normalized to the internal control pRL-TK and calculated as the fold induction compared with control cells transfected but not stimulated. The data represent the mean  $\pm$  SE from a total of five independent experiments. **C**, IL-17A mediates STAT3 but not STAT6 phosphorylation in human ASM cells. Growth-arrested ASM cells were stimulated with IL-17A or medium for 10 min. Total cell lysates were analyzed with anti-phospho-STAT3 (P-STAT3) or anti-phospho-STAT6 (P-STAT6) Abs. The same blot was stripped and reprobed with anti-STAT3 (STAT3) or STAT6 (STAT6). The results represent one of similar results from three independent experiments.



(U 0126), the JNK inhibitor II (SP600125), JAK2 inhibitor II, and JAK3 inhibitor VI were purchased from Calbiochem. Mouse IgG isotype control (clone MOPC21), were from Sigma-Aldrich Canada. DMEM, Ham's F-12 medium, antibiotics (penicillin, streptomycin), and FBS were purchased from HyClone Laboratories, and, unless stated otherwise, all other reagents were obtained from Sigma-Aldrich.

#### Preparation and culture of bronchial human ASM cells

Bronchial human ASM cells were obtained from macroscopically healthy segments of the main bronchus after lung resection from surgical patients in accordance with procedures approved by the Human Research Ethics Board of the University of Manitoba (Winnipeg, Canada). Primary ASM cell cultures were established and performed as described previously (13, 14).

#### Luciferase reporter constructs and cell transfection

Plasmids expressing a dominant negative (DN) form of STAT3 (pSG5hSTAT3 $\beta$ ) and a STAT3 mutant at serine 727 (S727A) were donated by Dr. R. Fostra (Royal Cancer Hospital, Brampton, U.K.) and Dr. R. Jove, (H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL), respectively. A Stat3-specific reporter plasmid (pLucTKS3), which harbors seven copies of a sequence corresponding to the STAT3-specific binding site in the C-reactive gene promoter (termed APRE; TTCC CGAA), and pLucSRE, which contains two copies of the serum response element of the *c-fos* promoter, both upstream from a firefly luciferase coding sequence (15) were donated by Dr. J. Turkson (University of Central Florida College of Medicine, Orlando, FL). STAT6 DN and eotaxin-1/CCL11-wild type (WT), STAT6 (M1), NF- $\kappa$ B (M3), and a double mutant (M2) promoter luciferase construct was provided by Dr. J. Hoeck (Salzburg University, Salzburg, Austria) (16). IL-6 promoter was provided by

Dr. O. Eickelberg (University of Giessen School of Medicine, Giessen, Germany). ASM cells ( $4 \times 10^4$ ) were seeded into 12-well culture plates in fresh complete DMEM. After 24 h at 50–70% confluency, cells were transfected transiently using ExGen 500 in vitro transfection reagent (Fermentas) according to the manufacturer's instructions. In each well, 1.6  $\mu$ g of promoter-luciferase DNA and 0.4  $\mu$ g of *Renilla* luciferase reporter vector pRL-TK (Promega) were cotransfected for 24 h. In some experiments, 0.8  $\mu$ g of DN STAT3, STAT6, or STAT3 Ser 727 mutant were cotransfected with WT CCL11 promoter-luciferase DNA (0.8  $\mu$ g). The medium was changed and cells were washed and stimulated with IL-17A or IL-4 (both at 10 ng/ml). After 12 h of cytokine stimulation, cells were washed twice with PBS and cell lysates were collected with 100  $\mu$ l of reporter lysis buffer (Promega). The luciferase activity was measured by the Dual-Luciferase assay system kit (Promega) using a luminometer (model LB9501; Berthold Lumat) as we described previously (17). All values are normalized to *Renilla* luciferase activity and expressed relative to the control-transfected nonstimulated cells.

#### Assessment of STAT3, STAT5, and STAT6 phosphorylation

Nearly confluent ASM cells were growth arrested by FBS deprivation for 48 h as described above. Cells were then stimulated in fresh FBS free medium with IL-17A (10 ng/ml), IL-4 (10 ng/ml), or medium alone. For pharmacological studies, cells were pretreated with inhibitors for ERK1/2 (U0126; 10  $\mu$ M), p38 (SB203580; 10  $\mu$ M), JNK1/2 (SP600125; 40 nM), JAK2 inhibitor II (10  $\mu$ M), and JAK3 inhibitor VI (10  $\mu$ M) before stimulation with IL-17A (10 ng/ml) or IL-4 (10 ng/ml). Concentration of MAPK inhibitors are based on our study (13, 17) and others (18). The concentration of JAK3 inhibitor VI was chosen as multiples of the in vitro IC<sub>50</sub> as provided by the manufacturer. The concentration of JAK2 inhibitor II was determined by the concentration for maximum inhibition (50  $\mu$ M).

as provided by the manufacturer and decreased to the maximum amount, showing no apparent toxicity in our cell system (10  $\mu$ M). Western blotting with Abs specific for phosphorylated and total STAT3, STAT5, or STAT6 was performed as described previously (13).

#### Chromatin immunoprecipitation (ChIP) assay

A ChIP assay was done essentially as described elsewhere, with minor modifications (19). Briefly, human ASM cells in 90-mm dishes were grown to confluency and serum starved for 48 h. Cells were then incubated with or without IL-17A or IL-4 (both at 10 ng/ml) for 1 and 2 h. The cells were cross-linked for 10 min with 1% formaldehyde in PBS to fix protein-DNA complexes, washed, and lysed and sonicated on ice. Samples were pre-cleared with salmon sperm DNA-saturated protein A-agarose (Upstate Biotechnology) for 1 h at 4°C with rotation. After centrifugation, one portion of the soluble chromatin was used as DNA input control, and the remains were subaliquoted and then precipitated using specific Abs, a nonimmune rabbit IgG (Santa Cruz Biotechnology), STAT6, or STAT3 rabbit mAb separately overnight at 4°C. The DNA from sample and input was amplified by semiquantitative PCR (30 cycles) using the primers specific for the CCL11 promoter (−136 to +61) encompassing both STAT6 and NF- $\kappa$ B binding sites. Primer pairs of CCL11/promoter are 5'-CTTCATGTTGG AGGCTGAAG-3' (forward) and 5'-GGATCTGGAATCTGGTCAGC-3' (reverse). PCR products were resolved by using 3% agarose gel and visualized with ethidium bromide.

#### Inhibitory peptide

Peptides used in this study were described previously (20). A cell-permeable STAT3 peptide inhibitor, which consists of H-PY\*<sup>1</sup>LKTK-AAVL LPVLLAAP-OH, and a control peptide, H-PYLKTK-AAVLLPVLLAAP-OH, were used. The control peptide is identical in sequence to the inhibitor except that the tyrosine residue, which is essential for the inhibitory action, is not phosphorylated (20). The peptides were synthesized by Bio S&T. Peptides were used at concentrations of 500  $\mu$ M (20).

#### Lentiviral vector transduction in human ASM cells

293T cells used for virus production and titration were cultured in IMDM (HyClone Laboratories) supplemented with 10% FBS and 1% penicillin/streptomycin/glutamate (PSG) (Invitrogen). Lentiviruses were generated and titrated using 293T cell lines as previously described (21). For gene silencing, two clones specific for STAT3 (clone identifiers V2LHS\_262105 and V2LHS\_88502) and one clone specific for STAT6 (clone identifier V2LHS\_153578) were obtained from Open-Biosystems. A control-scrambled short hairpin RNA (ShRNA) unrelated to STAT3 or STAT6 sequences was used as a negative control for lentiviral transduction. For all gene silencing studies, ASM cells were transduced at 10 infectious units per milliliter in the presence of Polybrene (8  $\mu$ g/ml). In brief, cells were exposed to recombinant lentivirus for 2 h at 37°C, the medium was replaced, and the cells were cultured for additional 72 h. Transduced cells were selected with puromycin (4  $\mu$ g/ml).

#### RNA isolation and real-time RT-PCR analysis

Confluent ASM cells stably transduced with lentiviral vector expressing STAT3 or scramble ShRNA were growth arrested for 48 h in serum-free medium as described above. Cells were then stimulated in fresh FBS-free medium containing human rIL-17A, IL-4, (both at 10 ng/ml), or vehicle (medium alone) for 6 h. Cells were harvested and total cellular RNA was extracted using the guanidinium isothiocyanate method (22). Relative levels of CCL11 mRNA were analyzed using quantitative real-time PCR analysis by LightCycler (Roche) as we previously described (13).

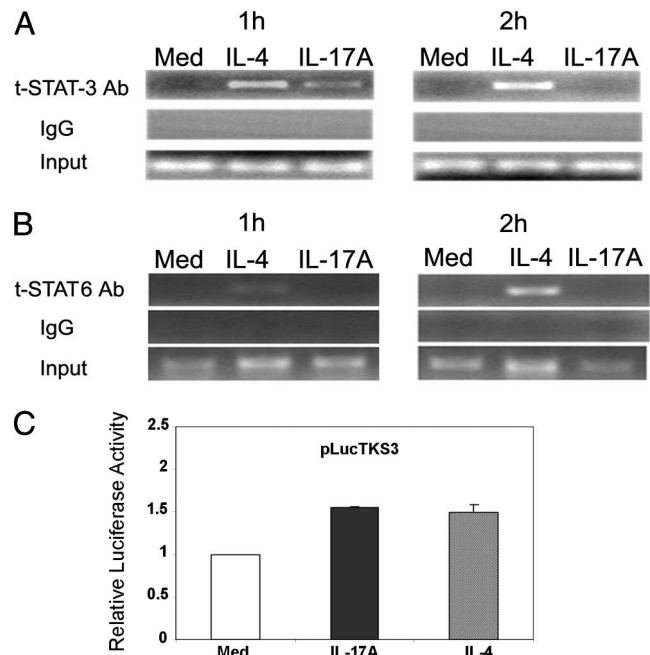
#### Statistical analysis

Results are expressed as means  $\pm$  SD. Differences between the groups were analyzed using Kruskal-Wallis with Dunn's test. Differences between pairs were assessed by the Mann-Whitney *U* test. Values of  $p < 0.05$  were considered statistically significant.

## Results

#### IL-17A-mediated CCL11 promoter activity requires a STAT6 binding site

We first determined whether IL-17A-mediated CCL11 expression depends on STAT6 and/or NF- $\kappa$ B binding to consensus DNA elements in the 5' promoter. ASM cells were transiently transfected with constructs harboring either a WT 2.2-kb CCL11 promoter sequence or three different mutation constructs that have specific



**FIGURE 2.** IL-17A induced STAT3 but not STAT6 binding to the CCL11 promoter in vivo. *A* and *B*, Confluent and serum-starved human ASM cells were treated with IL-4 (10 ng/ml) or IL-17A (10 ng/ml) for 1 and 2 h. In vivo STAT3 (*A*) or STAT6 (*B*) binding to the CCL11 promoter was analyzed by ChIP assay as described under *Experimental Procedures*. The input represents PCR products from chromatin pellets before immunoprecipitation. The results are representative of three independent experiments with similar results. t-STAT-3 Ab, Total STAT3 Ab; Med, medium. *C*, IL-17A-driven STAT3 reporter gene activity. The STAT3-specific reporter plasmid (pLucTKS3), which harbors seven copies of a sequence corresponding to the STAT3-specific binding site, or the pLucSRE serum response element of the *c-fos* promoter (data not shown) was transfected into ASM cells following by stimulation by IL-17A or IL-4 as described in Fig. 1*B*. Data represent the mean  $\pm$  SE from a total of five independent experiments.

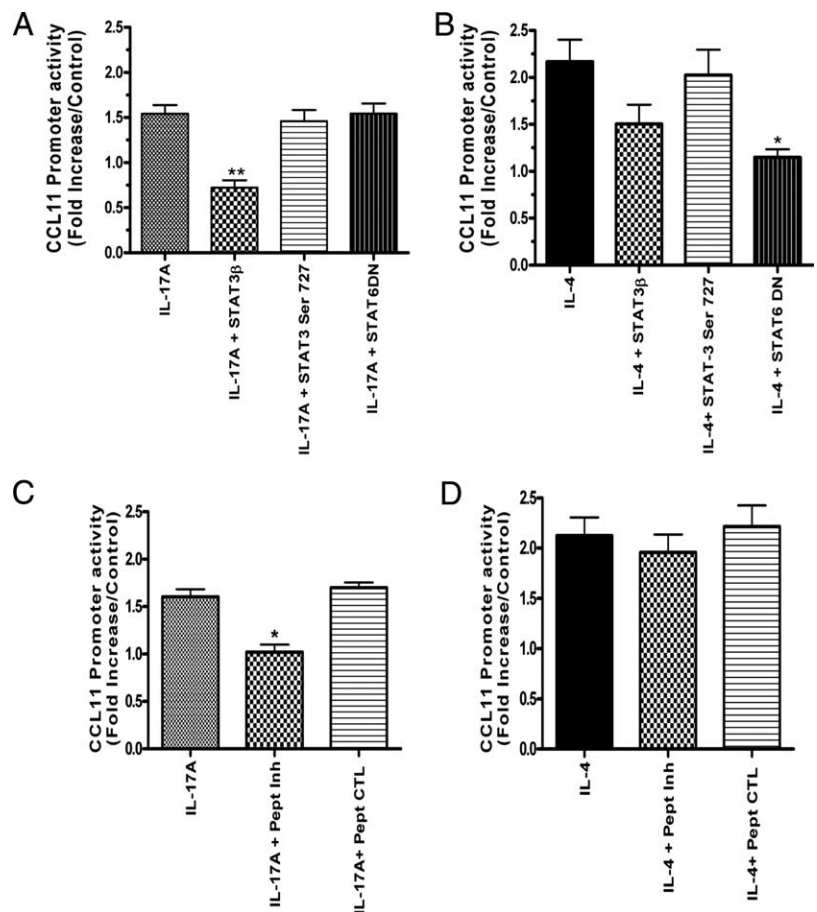
disruptions to regions for the binding of STAT6 and or NF- $\kappa$ B (16). The M1 construct contained a mutation that disrupts the STAT6 site found outside the NF- $\kappa$ B binding sequence. The M2 construct enclosed a mutation in an overlapping portion of STAT6 and NF- $\kappa$ B elements. The M3 construct harbored only a mutation in NF- $\kappa$ B binding site that does not overlap with the STAT6 core element (16) (Fig. 1*A*). Luciferase activity in response to IL-4 and IL-17A was significantly reduced for both the M1 (STAT6) and M2 (STAT6/NF- $\kappa$ B) constructs compared with the WT ( $p < 0.01$ ; Fig. 1*B*). In contrast, the M3 construct, the NF- $\kappa$ B specific mutation, retained a normal and an enhanced response to IL-17A and IL-4, respectively ( $n = 5$ ; Fig. 1*B*). These results suggest that IL-17A-mediated CCL11 expression requires a STAT6 binding site. Interestingly, as we showed previously (13), IL-17A induced an increase in STAT3 phosphorylation in ASM cells, but no STAT6 or STAT5 phosphorylation was observed (Fig. 1*C*). Altogether, our data suggest that IL-17A activates STAT3, which may bind to the STAT6 binding site within the CCL11 promoter.

#### IL-17A induced STAT3 but not STAT6 binding to CCL11 promoter in vivo

To detect whether STAT3 or STAT6 binds in vivo to the CCL11 promoter following IL-17A stimulation, a ChIP assay coupled to PCR was performed. We also determined the status of the STAT3 or STAT6 transcription complex at the human CCL11 promoter in response to IL-4 as a positive control. Equal amounts of nuclear



**FIGURE 3.** STAT3 DN (STAT3 $\beta$ ) and cell-permeable STAT3 peptide inhibitor (Pept inh), but not STAT3 Ser 727, inhibits CCL11 promoter activity induced by IL-17A in ASM cells. *A* and *B*, Human primary ASM cells were cotransfected with WT CCL11 promoter and DN STAT3, STAT3 $\beta$ , STAT3 mutant at Ser 727, or DN STAT6. Twenty-four hours after transfection, cells were stimulated for 12 h with IL-17A (*A*) or IL-4 (*B*) (both at 10 ng/ml), after which transcriptional activation was measured by luciferase activity. *C* and *D*, Effect of STAT3 inhibitory peptide, PY\*LKTK-mts, on IL-17A-mediated CCL11 transcriptional activity. Human primary ASM cells were growth arrested and transfected with the CCL11 promoter for 24 h. Cells were then incubated with PY\*LKTK for 1 h before stimulation with IL-17A (*C*) or IL-4 (*D*) as described above. Fold induction represents luciferase activity in cytokine-treated cells compared with untreated cells and is the mean of five independent experiments. \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ .



samples were immunoprecipitated with an Ab against STAT3 or STAT6, and the immunoprecipitated DNA was amplified with the specific human CCL11 primer pairs. In contrast to unstimulated cells, IL-17A induced at 1 h a marked enrichment of STAT3-associated CCL11 promoter DNA, a 197-bp region between  $-136$  and  $+61$ , which returned to baseline at 2 h (Fig. 2A). In contrast, immunoprecipitation with a nonspecific Ab resulted in the absence of a 197-bp band, suggesting that STAT3 specifically binds to the CCL11 promoter. Interestingly, IL-4 treatment resulted in a clear enrichment of both STAT3 and STAT6 at 1 and 2 h (Fig. 2, A and B) that returned to the baseline at 4 h (data not shown). Furthermore, no STAT6-associated CCL11 promoter DNA band could be detected in IL-17A-stimulated ASM cells. These data suggest that IL-17A-induced CCL11 transcription involves *in vivo* STAT3 binding to the CCL11 promoter.

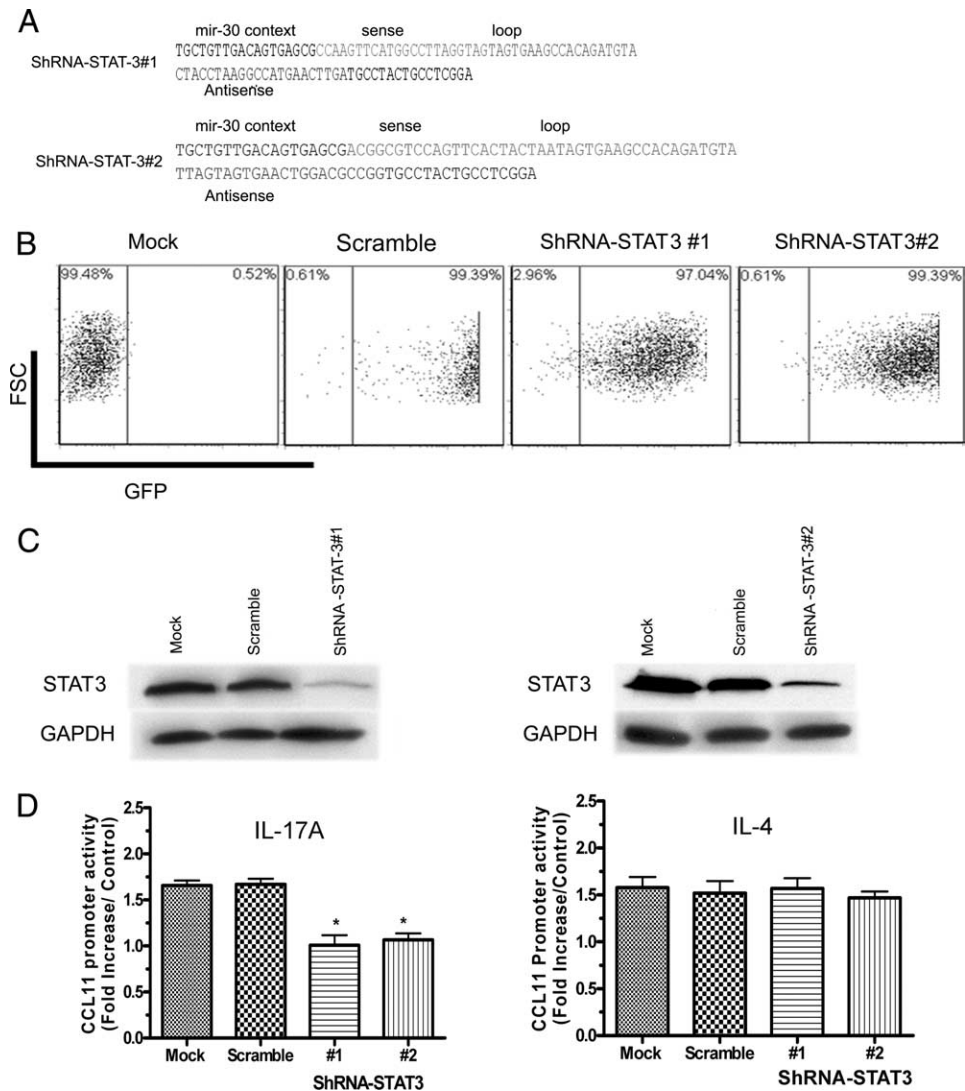
We next used a STAT3-specific reporter plasmid (pLucTKS3) that harbors seven copies of a sequence corresponding to the STAT3-specific binding site in the C-reactive gene promoter (15) to confirm the activation of STAT3-mediated gene expression by IL-17A. As shown in Fig. 2C, both IL-17A and IL-4 are able to drive STAT3-dependent luciferase promoter activity compared with transfected unstimulated ASM cells (mean relative luciferase units (RLU)  $\pm$  SD was  $62,458 \pm 13,948$  for IL-17A,  $59,316 \pm 9,458$  for IL-4, and  $40,231 \pm 8,766$  for medium;  $p < 0.05$ ,  $n = 5$ ). Furthermore, IL-17A and IL-4 are not able to induce luciferase promoter activity when cells are transiently transfected with pLucSRE serum response element of the *c-fos* promoter (mean RLU  $\pm$  SD was  $122 \pm 32$  for IL-17A,  $125 \pm 15$  for IL-4, and  $98 \pm 23$  for medium;  $n = 5$ ,  $p > 0.05$ ).

#### STAT3 DN, STAT3 $\beta$ , and cell-permeable STAT3 inhibitory phosphopeptide reduces CCL11 promoter activity induced by IL-17A

To confirm the role of STAT3 in IL-17A-mediated CCL11 promoter activity, we used two approaches. We first disrupted STAT3 function by using STAT3 DN proteins that interfere with STAT3 signaling (15). ASM cells were transiently cotransfected with a luciferase reporter construct driven by a 2.2-kb CCL11 proximal promoter and a construct overexpressing a DN STAT3, pSG5hSTAT3 $\beta$  (15). STAT3 $\beta$  is a naturally occurring splice variant with a deletion in the C-terminal *trans*-activation domain that harbors the transcriptional activation domain and the Ser<sup>727</sup> residue (23, 24). Cells cotransfected with a CCL11 promoter construct and a STAT3 $\beta$ -expressing vector showed a significant decrease in luciferase activity in response to IL-17A ( $p < 0.01$ ,  $n = 5$ ; Fig. 3A). In contrast to STAT3 $\beta$ , cotransfection with DN STAT6 did not affect promoter induction by IL-17A (Fig. 3A). As expected, cotransfection with DN STAT6 significantly decreases IL-4-induced CCL11 promoter activity ( $p < 0.05$ ;  $n = 5$ ). Furthermore, STAT3 $\beta$  displayed a moderate effect on CCL11 promoter activity induced by IL-4 but was not statistically significant ( $p > 0.05$ ,  $n = 5$ ).

In a second approach, we pretreated ASM cells with a STAT3 phosphopeptide, a cell-permeable inhibitor that acts as a highly selective blocker of STAT3 activation (20). The STAT3 control peptide has the same residue sequence without the phospho group. ASM cells were transiently transfected with the CCL11 proximal promoter and pretreated with the STAT3 inhibitory peptide followed by IL-17A stimulation for 12 h. Human ASM

**FIGURE 4.** Silencing STAT3 abrogates IL-17A-mediated CCL11 expression. **A**, Sequence of the two clones used for silencing STAT3 in this study. **B**, Efficiency of lentiviral transduction in human ASM cells. Human ASM cells were transduced by infecting with lentivirus containing scramble sequence or STAT3-ShRNA sequence and examined by flow cytometry for tGFP expression. **C**, Effect of STAT3-ShRNA on STAT3 expression by ASM cells. Expression of total STAT3 in mock, scramble, and STAT3-ShRNA-transduced ASM cells was analyzed by Western blotting. **D**, ASM cells stably expressing scramble or STAT3-ShRNA was transfected with WT CCL11 promoter luciferase reporter plasmid and stimulated with IL-17A (10 ng/ml; *left*) or IL-4 (10 ng/ml; *right*) as described in *Materials and Methods*. The mean  $\pm$  SE of three independent experiments is shown. \*,  $p < 0.05$  compared with scramble lentiviral transduced ASM cells stimulated with IL-17A.



cells showed a significant reduction ( $p < 0.05$ ) in CCL11 luciferase activity in response to IL-17A stimulation following STAT3 inhibition with the STAT3 inhibitory peptide (Fig. 3C). No effect could be observed in STAT3 control peptide-treated cells. STAT3 inhibitory peptide did not affect IL-4 induced CCL11 promoter activity in ASM cells (Fig. 3D). We also examined the effect of this peptide inhibitor on STAT3 and STAT6 phosphorylation in IL-17A-stimulated ASM cells. In contrast to the STAT3 control peptide, the STAT3 inhibitory peptide inhibits STAT3 phosphorylation at 20 min but does not affect STAT6 phosphorylation or ERK1/2 (data not shown). These results confirm that STAT3 activation is involved in IL-17A-induced CCL11 promoter activity.

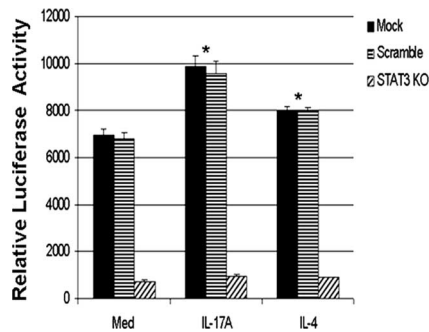
#### STAT3 mutant at Ser<sup>727</sup> is not required for IL-17A-induced CCL11 expression

Depending on the cell system, STAT3 Ser<sup>727</sup> mutation has been shown to either have no effect (23) or to enhance or down-regulate (25) the transcriptional activity of target genes. We then decided to investigate its role in IL-17A-induced CCL11 promoter induction. Interestingly, cotransfection of ASM cells with mutant STAT3 Ser<sup>727A</sup> exhibited no effect on CCL11 promoter activity induced by IL-17A stimulation (Fig. 3A). Similarly, induction of CCL11 by IL-4, used as positive control,

also was not affected by STAT3 Ser<sup>727</sup> cotransfection ( $n = 5$ ; Fig. 3B).

#### ShRNA-mediated STAT3 silencing abrogates IL-17A-mediated CCL11 expression

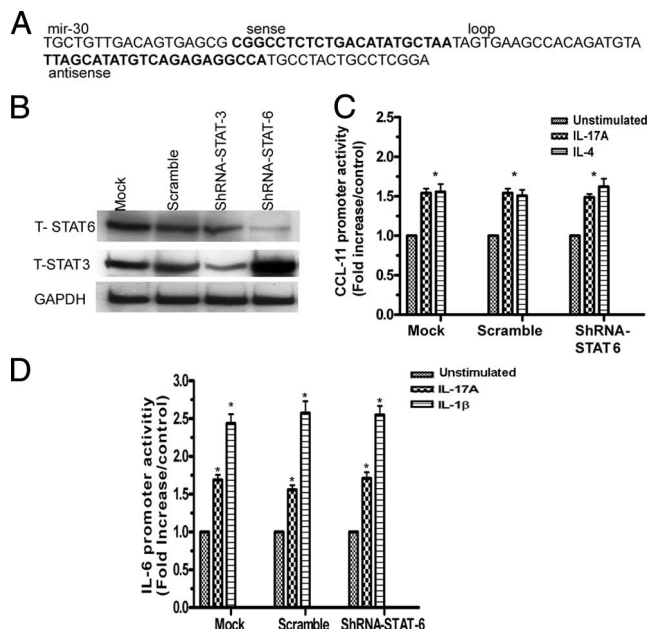
To inhibit STAT3 expression in ASM cells, two double-stranded ShRNA clones of human STAT3 were tested for their efficiency to shut down STAT3 in ASM cells (Fig. 4A). Mock and scramble sequences were used as negative controls. The transduction efficiency of ASM cells was monitored by FACS analysis using the vector turboGFP (tGFP) reporter gene. As revealed by FACS analysis,  $> 95\%$  of the lentiviral transduced cells were tGFP<sup>+</sup> (Fig. 4B). Transduction of cells with STAT3-ShRNA clones (Fig. 4B) resulted in a highly significant and reproducible decrease in STAT3 expression levels as shown by Western blotting (Fig. 4C). As expected, transduction with scramble ShRNA failed to reduce STAT3 expression in ASM cells. To determine whether the transcriptional activation of CCL11 by IL-17A is affected in the absence of STAT3, stably STAT3-silenced ASM cells were transfected with CCL11 promoter and stimulated with IL-17A (10 ng/ml) or IL-4 (10 ng/ml). As observed in Fig. 4D, IL-17A-induced transcriptional activation of CCL11 promoter was significantly reduced in STAT3-ShRNA-transduced cells but unchanged in scramble-transduced cells ( $n = 5$ ,  $p < 0.05$ ). Furthermore, CCL11



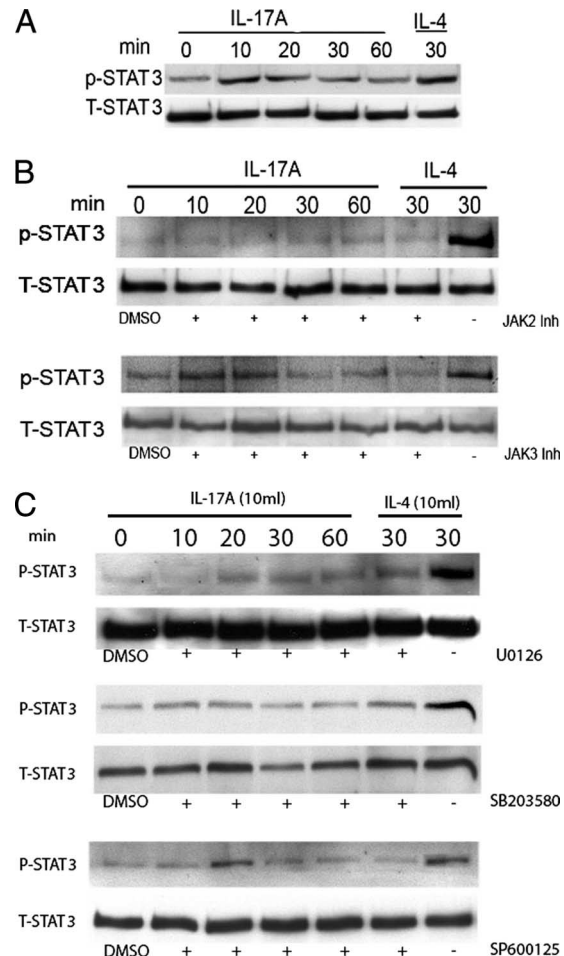
**FIGURE 5.** IL-17A-driven STAT3 reporter gene activity is abrogated in STAT3-silenced ASM cells. STAT3-specific reporter plasmid (pLucTKS3), which harbors seven copies of a sequence corresponding to the STAT3-specific binding site, was transfected into WT cells or STAT3-, or scramble-silenced ASM cells following by IL-17A or IL-4 stimulation as described in *Material and Methods*. Data represent the mean  $\pm$  SE from a total of five independent experiments. \*,  $p < 0.001$  compared with STAT3-silenced ASM cells. KO, Knockout.

promoter activity induced by IL-4 was unaffected in STAT3-silenced ASM cells (Fig. 4D). Similarly, STAT3-silenced ASM cells exhibited reduced CCL11 mRNA expression following IL-17A but not IL-4 stimulation (data not shown).

We then transfected the STAT3 reporter gene, pLucTKS3, into WT, scramble, or STAT3-silenced ASM cells to validate the role of IL-17A in driving gene expression in a STAT3-dependent man-



**FIGURE 6.** IL-17A-mediated CCL11 expression is not affected in STAT6-silenced ASM cells. **A**, Sequence of STAT6-ShRNA used in this study. **B**, Effect of STAT6-ShRNA on STAT3 expression by ASM cells. Human ASM cells were transduced by infection with lentivirus containing scramble sequence, STAT6-ShRNA sequence, or mock and examined by flow cytometry for GFP expression. Expressions of total STAT6 and STAT3 in mock-, scramble-, STAT3-ShRNA-, or STAT6-ShRNA-transduced ASM cells were analyzed by Western blotting. **C** and **D**, Stably silenced STAT3 ASM cells were cotransfected with CCL11 promoter (**C**) and IL-6 promoter (**D**) luciferase reporter plasmid, respectively, after transfection, cells were stimulated with IL-17A, IL-4, or IL-1 $\beta$  (all at 10 ng/ml) as described in *Materials and Methods*. The mean  $\pm$  SE of three independent experiments are shown. \*,  $p < 0.05$  compared with CCL11 luciferase promoter-transfected but not -stimulated ASM cells.



**FIGURE 7.** ERK1/2 and JAK2 pharmacological inhibitors abolish IL-17A-mediated STAT3 phosphorylation. Growth-arrested ASM cells were pretreated with pharmacological inhibitors (Inh) of MAPK (**A**), JAK2 (Inh II) or JAK3 (Inh VI) (**B**), and ERK1/2 (U0126), p38 (SB203580), or JNK (SP600125) (**C**) and then stimulated with IL-17A and IL-4, both at 10 ng/ml, as described in *Materials and Methods*. Total cell lysates were analyzed with anti-phospho STAT3 (p-STAT-3). The same blot was stripped and reprobed with anti-STAT3 (T-STAT-3). The results represent one of similar results from three independent experiments.

ner. STAT3-dependent luciferase promoter activity driven by IL-17A or IL-4 was reduced dramatically by 10- and 9-fold, respectively, in STAT3-silenced ASM cells compared with WT cells (in WT and STAT3-silenced cells, RLU was  $9,572 \pm 550$  vs  $947 \pm 32$ , respectively, for IL-17A, and  $8007 \pm 125$  vs  $873 \pm 7$ , respectively, for IL-4; Fig. 5). Collectively, these results confirm the role of STAT3 activation in IL-17A-induced CCL11 expression.

#### IL-17A mediates CCL11 expression in STAT6-silenced ASM cells

We then evaluated whether STAT6 silencing affects IL-17A-mediated CCL11 expression in ASM cells. Transduction of ASM cells with vector harboring STAT6-ShRNA or scramble ShRNA showed that  $> 95\%$  of ShRNA-transduced cells were tGFP<sup>+</sup> (data not shown) and resulted in a highly significant and reproducible decrease in STAT6 expression levels (Fig. 6B). As expected, transduction with scramble ShRNA did not reduce STAT6 expression in ASM cells. To determine whether the transcriptional activation of CCL11 by IL-17A is affected in the absence of STAT6, stably silenced STAT6 ASM cells were transfected with CCL11 promoter and stimulated with IL-17A (10 ng/ml) or IL-4 (10 ng/ml).



IL-17A-induced transcriptional activation of CCL11 promoter was unchanged in STAT6-silenced transduced cells or in scramble-silenced cells ( $n = 3$ ,  $p < 0.05$ ). Surprisingly, CCL11 promoter activity induced by IL-4 was unaffected in STAT6-silenced ASM cells (Fig. 6C). To investigate the specificity of silencing STAT6 in ASM cells, we analyzed promoter activity of IL-6 luciferase construct, a gene that is induced by IL-17A in various structural cells (26, 27) but is not dependent on the STAT6 pathway. IL-6 promoter activity was comparable in mock-, scramble-, and STAT6-silenced ASM cells following IL-17A or IL-1 $\beta$  stimulation ( $n = 3$ ; Fig. 6D).

Collectively, these results validate the role of STAT3 but not STAT6 activation in IL-17A-induced CCL11 expression.

#### *ERK1/2 and JAK2 pharmacological inhibitors abolish IL-17A-mediated STAT3 phosphorylation*

Previously, we showed that blocking MAPKs (ERK1/2, p38, and JNK) and JAK diminish significantly IL-17A-mediated CCL11 release (13). To elucidate whether cross-talk between MAPKs or the JAK and STAT3 pathways is occurring in IL-17A-mediated CCL11 expression, pharmacological inhibitors of MAPKs (ERK1/2, p38, and JNK) and JAK2 and JAK3 were used. As shown in Fig. 7, B and C, we found that ERK1/2 (U0126) and JAK2 have a profound effect on STAT3 phosphorylation. No clear significant effect could be detected with p38 and JNK or JAK3. IL-4-induced STAT3 phosphorylation was significantly reduced by inhibitors of JAK2, JAK 3, ERK1/2, and JNK ( $n = 3$ ; Fig. 7). We did not test JAK1 because there is no commercially available specific inhibitor for JAK1. Taken together, our data suggest that IL-17A-mediated STAT3 activation is downstream of JAK2 and ERK1/2 signaling pathways.

## Discussion

Previously, we showed that IL-17A can induce CCL11 mRNA expression and protein release from ASM cells (13). This effect of IL-17A is dependent on de novo protein and mRNA synthesis and activation of MAPK-signaling pathways. In this study, we aimed to define the involvement of STAT3 in IL-17A-mediated CCL11 expression in ASM cells. Using genetic and biochemical approaches, we demonstrated that IL-17A-mediated CCL11 expression in ASM cells requires STAT3 activation. Mutation of the STAT binding site but not NF- $\kappa$ B abolishes CCL11 promoter activity induced by IL-17A. A ChIP assay showed binding of STAT3 to the CCL11 promoter in vivo. Overexpression of naturally occurring DN STAT3 $\beta$ , which lacks a *trans*-activation domain (23, 24), but not Ser 727 STAT3 or STAT6 DN, in ASM cells abolishes IL-17A-mediated CCL11 promoter activity. Disruption of STAT3 activity with SH2 domain binding peptide (PY\*LKTK-mts), but not with control peptide (PYLKTK-mts) (where mts is membrane-translocating sequence), results in a significant reduction of IL-17A-mediated STAT3 phosphorylation and CCL11 promoter activity. STAT3- but not STAT6-silenced ASM cells showed significant reduction in IL-17A-mediated CCL11 promoter activity and mRNA expression. Finally, we found that inhibitors of ERK1/2 or JAK2 abolishes IL-17A-mediated STAT3 activation in ASM cells.

IL-17A, a pleiotropic T lymphocyte cytokine released from a distinctive Th1/Th2 lineage subset (28), is hypothesized to orchestrate the granulocyte influx into the airways via the induction of chemokines (27, 29). A potential role of IL-17A in the inflammatory response is suggested by elevated IL-17A expression in mononuclear cells from patients with multiple sclerosis (7), rheumatoid arthritis (30), or systemic lupus erythematosus (31). Furthermore, elevated IL-17A levels can be detected in sera, sputum,

and bronchoalveolar lavage fluid from asthmatic patients and have been shown to correlate with airway hyperresponsiveness (9, 10, 26, 32, 33). In allergic asthma, a direct role of IL-17A signaling in airway recruitment of eosinophils has been demonstrated in a mouse model (34). In fact, IL-17R-deficient mice displayed reduced lung recruitment of granulocytes, predominantly eosinophils (34). Similarly, transgenic mice overexpressing IL-17A within the airways showed a clear induction of CCL11 and eosinophils. The current study extends these results by clearly showing that IL-17A-mediated CCL11 in ASM cells uses STAT3 activation and not STAT6 activation, thus revealing a new pathway for induction of CCL11 within the airways.

The promoter of eotaxin-1/CCL11 contains response elements for NF- $\kappa$ B, AP1, C/EBP, and STAT6 binding sites (16). In our study, IL-17A- as well as IL-4-mediated CCL11 expression was negatively affected by STAT6 mutation at the CCL11 promoter. However, a significant increase in STAT3 but not STAT6 phosphorylation was detected following IL-17A stimulation. Although STAT6-mediated transcriptional activity without phosphorylation may happen in ASM cells, as has been shown for STAT 1 in some cell lines, a ChIP assay clearly showed in vivo binding of STAT3, but not STAT6, to the CCL11 promoter following IL-17A stimulation. Furthermore, similar to our findings, STAT3-dependent CCL11 expression has been shown in oncostatin M-stimulated human ASM cells (35) and mouse fibroblasts (36). Taken together, our data showed for the first time the involvement of IL-17A-mediated gene expression via a STAT3-dependent pathway.

We also found that IL-4 can induce both STAT3 and STAT6 binding to the CCL11 promoter in ASM cells. This raises the possibility that IL-4 can use STAT3 and STAT6 in ASM cells as previously demonstrated in normal human B cells (37) and CD8<sup>+</sup> T cells (38). This also may explain that in STAT6 knockout cells IL-4 mediated CCL11 activation was not affected (Fig. 6C), because STAT3 phosphorylation can be induced by IL-4 (Fig. 7) and mediates CCL11 promoter activity. Interestingly, upon IL-4 stimulation an NF- $\kappa$ B-mutated CCL11 promoter construct showed enhanced promoter activity. In the absence of NF- $\kappa$ B binding, STAT3 may promote chromatin accessibility and allow other transcription factors to mediate gene expression as described for STAT4 (39) or, alternatively, the prevention of DNA methyltransferase recruitment (40).

STAT3 is a member of a group of cytokine and growth factor-inducible transcription factors that have essential roles in the regulation of different types of immune and inflammatory responses (11). Once phosphorylated at tyrosine residues, STAT3 undergoes conformational change, dimerizes, and translocates to the nucleus. Within the nucleus, STAT3 binds specific DNA motifs and activates the transcription of distinct groups of genes (11). Cell-specific disruption of STAT3 in endothelial or myeloid cells suggests mainly an anti-inflammatory role of STAT3 in innate immune response (41). Additional evidence for a central role of STAT3 in regulating the anti-inflammatory response comes from skin and liver pathological models (41, 42). However, recent evidence clearly showed a key role of STAT3 in allergic inflammation. Disruption of STAT3 in airway epithelial cells (e-STAT3<sup>-/-</sup>) showed a significant decrease of airway eosinophilia (43), which is in agreement with our present data showing that CCL11 induced by IL-17A in ASM cells is dependent on STAT3. In our study, we found that overexpression of the STAT3 Ser<sup>727</sup> mutation in ASM cells has no effect on CCL11 promoter activity. This result is in agreement with data obtained in transgenic mice expressing a form of the STAT3 Ser<sup>727</sup> mutant (44) and in vitro using transfected cells with the STAT3 Ser<sup>727</sup> mutant (23). In both cases, tyrosine phosphorylation of STAT3 was detected in fibroblasts induced by



oncostatin M (44) or EGF-stimulated COS cells (23). Furthermore, in the serine-mutant mouse, many of the downstream signaling pathways associated with STAT3 remain intact (44), and phosphorylation on serine 727 has no effect on the DNA binding affinity of STAT3 (23). However, serine 727 may play a role by interacting with other cellular signaling pathways as described in keratinocytes (25, 44). Collectively, our data suggest that STAT3 serine phosphorylation has no effect on transcription of the CCL11 gene in ASM cells.

IL-17A can activate all three classes of MAPK: extracellular signal-regulated kinases (ERK); c-Jun N-terminal kinases (JNK); and p38 (14). Furthermore, the MAPKs have been shown to play an important role in modulating STAT3 signaling. Our data showed that pharmacological inhibition of ERK1/2, but not of p38 MAPK or JNK1/2, abolishes STAT3 tyrosine phosphorylation. Because we previously demonstrated that IL-17A-activated ERK1/2 (13) and IL-17A-mediated CCL11 promoter activities are significantly reduced by ERK1/2 (data not shown), it is reasonable to suggest that ERK1/2 regulates IL-17A-mediated STAT3 phosphorylation in ASM cells as has been previously demonstrated in the HEK293 cell line (45, 46). These results are in agreement with data where the ERK-MAPK pathway plays a dominant role in the IL-17A-elicited growth stimulatory effect on primary tracheal epithelial cells (47) or in IL-17A-induced IL-6 production in A549 pulmonary epithelial cells (48).

We also found that inhibition of JAK2 reduced dramatically STAT3 phosphorylation. Pharmacological targeting of the JAK pathway can reduce significantly CC11 release induced by IL-17A in ASM cells (13). Furthermore, JAK2 functions as an initiator of many signaling pathways, including the MAPK pathway (49). These observations may suggest that IL-17A signaling is initiated by JAK2 activation that affects ERK1/2 activation, leading to STAT3 tyrosine phosphorylation. Taken together, our data suggest a complex interaction and a regulatory cross-talk between the JAK2, ERK1/2, and STAT3 pathways in mediating IL-17A signaling in ASM cells.

In summary, this study is the first demonstration of the capacity for IL-17A to induce gene expression via the STAT3 pathway in ASM cells. Our results also provide a better understanding of IL-17A-ASM cell interactions and their role in airway inflammatory responses. Further work using an in vivo model is needed to demonstrate the contribution of this pathway to regulation of the inflammatory response.

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## Disclosures

The authors have no financial conflict of interest.

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