

# Der p, IL-4, and TGF- $\beta$ Cooperatively Induce EGFR-Dependent TARC Expression in Airway Epithelium

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Thymus and Activation-Regulated Chemokine (TARC) may be critical in Th2 cell recruitment in allergic inflammation; however, the mechanisms of allergen-induced TARC release are unclear. Since airway epithelium is the first line of defense to inhaled allergens, we questioned whether house dust mite allergen (Der p) can induce TARC expression in bronchial epithelial cells, how this is regulated at the molecular level, and if micro-environmental cytokines augment this effect. We examined the effects of Der p and the cytokines IL-4 and TGF- $\beta$  on TARC expression in 16HBE cells and primary bronchial asthma epithelium. Real-time PCR and immunofluorescence demonstrated that Der p induces TARC expression in bronchial epithelium. Supernatants from Der p-stimulated 16HBE cells were able to induce TARC-dependent T cell trafficking. IL-4 and TGF- $\beta$  cooperatively enhanced Der p-induced TARC expression in 16HBE cells. Specific inhibitors, immunodetection, and gel-shifts revealed that these effects are mediated by phosphorylation of the epidermal growth factor receptor (EGFR), mitogen-activated protein kinase (MAPK) signaling and subsequent nuclear factor (NF)- $\kappa$ B activation. A Disintegrin And Metalloproteinase (ADAM), a family of proteins involved in shedding of various growth factors, was shown to be responsible for EGFR activation. The increase in TARC production by direct interaction of Der p with the bronchial epithelium may be an important initial step in the generation of allergic inflammation, which is further potentiated by micro-environmental cytokines. Interference with ADAM or EGFR activity may be a novel promising target to prevent TARC release and subsequent allergic inflammation.

**Keywords:** human; chemokine; inflammation; signal transduction; lung

Allergic asthma is characterized by airway inflammation. In sensitized individuals, recruitment of T helper type 2 (Th2) cells and subsequent production of Th2-type cytokines like IL-4, IL-5, and IL-13 orchestrate the inflammatory response to inhaled aeroallergens. Cells of the innate immune system, especially airway epithelial cells, may play an important role in driving this Th2-mediated immune response. The airway epithelium is the first line of defense to inhaled allergens and may affect the outcome of the immune response by the production of various cytokines. These include Thymus and Activation-Regulated Chemokine (TARC) and thymic stromal lymphopoietin (TSLP). TSLP has been reported to prime dendritic cells to promote Th2 cell differentiation and may thus play a role in sensitization toward allergens (1), while TARC may be crucial for Th2 cell recruitment to asthmatic airways (2–6). The majority of Th2 cells express

## CLINICAL RELEVANCE

The concept of allergen-induced epithelial damage leading to TARC production and allergic inflammation is new in the field. The hitherto unrecognized role of ADAM/EGFR in Th2-mediated airway inflammation may lead to novel therapeutic approaches.

CCR4, the receptor for TARC. An allergen-induced increase in CCR4<sup>+</sup> T cells has been observed in asthmatic airways (7, 8). The mechanisms involved in allergen-induced airway inflammation and TARC release are largely unclear.

In asthma, the epithelial barrier is often disrupted and there is evidence for shedding of ciliated cells. This may be due to an inadequate repair response to damaging stimuli. Increased expression of repair mediators (e.g., epidermal growth factor receptor [EGFR] and TGF- $\beta$ ) has been observed at sites of ciliated cell detachment (9). Reduced barrier function may enhance access of allergens (e.g., house dust mite allergen/*Dermatophagoides pteronyssinus* [Der p]) to underlying antigen-presenting cells. Interestingly, increased permeability of the bronchial epithelium to Der p was accompanied by increased expression of various cytokines and NF- $\kappa$ B activity (10, 11). Several allergens, including Der p, cockroaches, fungi, and pollen, contain proteases that facilitate their passage over the epithelial layer through disruption of epithelial cell-cell contacts (12–16). Studies in mice have revealed that proteolytic activity is an important factor in the sensitization toward allergens (17, 18). This appears not only the result of disrupted barrier and facilitated access of allergens to antigen presenting cells (18), but proteases can activate epithelial cells (19–21) and induce the release of proinflammatory cytokines and chemokines as well (22). We were interested to examine if direct contact of the airway epithelium with aeroallergens induces TARC expression, thereby promoting Th2-driven immune responses. Furthermore, we questioned whether the induction of TARC might be augmented by proinflammatory micro-environmental factors. The effects of IL-4 and TGF- $\beta$  are of particular interest, given their prominent role in allergic airway inflammation and expression in asthmatic airways (23, 24). We analyzed the effect of Der p on TARC expression alone and in combination with IL-4 and TGF- $\beta$  and investigated the signaling pathways involved in the regulation of TARC expression in bronchial epithelial cells. We demonstrate that Der p, IL-4, and TGF- $\beta$  cooperatively induce TARC expression in bronchial epithelial cells. Our findings suggest that this is largely due to activation of EGFR, mitogen-activated protein kinase (MAPK) signaling, and subsequent NF- $\kappa$ B activation. Activity of A Disintegrin and Metalloproteinase (ADAM), a family of proteins that can induce ectodomain shedding of growth factors, appeared to be responsible for EGFR phosphorylation.

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## MATERIALS AND METHODS

### Cell Culture

Bronchial epithelial cells were obtained by bronchial brushing in nine subjects (40–73 yr) with mild asthma and atopy for house dust mite (HDM), according to standard guidelines (25). Patients were nonsmokers and free of other lung diseases, and had a baseline AMP PC<sub>20</sub> (the provocative concentration of AMP causing a 20% fall in FEV<sub>1</sub>) < 16 mg/ml. The Medical Ethics Committee of the University Hospital of Groningen approved the study and signed informed consent was given to participate. Primary cultures of bronchial epithelial cells were established as described previously (26). Cell cultures were cultured in 2.5 ml serum-free hormonally supplemented bronchial epithelium growth medium (Clonetics, San Diego, CA) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin coated with 30 µg/ml collagen, 30 µg/ml fibronectin, and 10 µg/ml bovine serum albumin (BSA). When 90% confluent, cells were passaged using trypsin and further cultured for use at passage 2. The human bronchial epithelial cell line 16HBE14o- was kindly provided by Dr. D.C. Gruenert, University of California, San Francisco. 16HBE cells were cultured on dishes coated with 30 µg/ml collagen and 10 µg/ml BSA in Eagle's Minimal Essential Medium (EMEM) (Biowhittaker, Verviers, Belgium) containing 10% fetal calf serum (FCS; Hyclone, Logan, UT) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. At 80–90% confluence, cells were passaged or used for experiments. Bronchial epithelial cells were seeded into 24-well plates at a density of 5 × 10<sup>4</sup>/well and again allowed to grow to 80–90% confluence. Medium was then replaced with basal medium (BEBM; Clonetics) for primary cultures or EMEM medium containing 0.5% FCS for 16HBE cells. The cells were rendered quiescent for 24 h before stimulation.

T cells from healthy individuals were isolated by density-gradient centrifugation and sheep red blood cell rosetting as described previously (27), and rested overnight in RPMI 1640 medium containing 1% FCS before experimentation. Signed informed consent was given to participate.

### Stimulation of Bronchial Epithelial Cells

Cells were pretreated for 30 min with the ADAM inhibitor TAPI-2 in a concentration of 22.5 µM (Calbiochem, Omnilabo International by, Breda, The Netherlands), the selective EGFR tyrosine kinase inhibitor AG1478 in a concentration of 1 µM (28, 29) (Sigma, St. Louis, MO), the selective MEK/ERK-1/2 inhibitor U0126 in a concentration of 10 µM (30) (Promega, Madison, WI) or 1 µM of the MAPK inhibitor SB203580, which is specific for p38 when used in this concentration (31) (SmithKline Beecham Pharmaceuticals, King of Prussia, PA) and subsequently exposed to 50 µg/ml of enzymatically active whole extracts of house dust mite (Der p), containing multiple components of Der p (a generous gift of Dr. L. Jakobsen, ALK, Copenhagen, Denmark) for 5, 20, 60 min, and 3, 6, or 24 h, in presence and absence of IL-4 (10 ng/ml; R&D Systems, ITK Diagnostics, Uithoorn, The Netherlands), TGF-β (2 ng/ml; Peprotech, Tebu-Bio, Heerhugowaard, The Netherlands). Nuclear extracts were prepared as described below after 3 h, RNA was extracted from cells by the TRIzol method (Gibco BRL, Burlington, ON, Canada) after 6 h, supernatants were harvested for T cell migration assays after 24 h, and cytopins were prepared after 3 or 24 h by trypsinization and spinning down.

### Real-Time RT-PCR

cDNA was synthesized as described previously (32). Expression of TARC mRNA was analyzed by quantitative real-time PCR using the Biorad MyiQ Single-Color detection system (Bio-Rad laboratories Inc., Life Science group, Hercules, CA) according to the manufacturer's guidelines. In short, 15 µl iQ SYBR Green Supermix, containing fluorescein to account for well to well variation, 0.1 µM of forward and reverse primer, and 5 µl of 1:5 diluted cDNA sample, were used in a total volume of 25 µl and added to a 96-well plate. The threshold cycle (Ct) of the Der p-stimulated 16HBE cells was compared with the Ct generated by the reference sample (the unstimulated 16HBE cells). Cytokine gene expression was normalized to expression of the house-keeping gene β<sub>2</sub>-microglobulin (β<sub>2</sub>µG), with approximately equal amplification efficiency. The ΔCt was calculated as the difference between the Ct values, determined using the equation 2<sup>-ΔCt</sup>. The following

specific primers pairs were obtained from Biogio BV (Malden, The Netherlands): β<sub>2</sub>µG, 5'CCAGCAGAGAATGGAAAGTC3' sense and 5'GATGCTGCTTACATGTCTCG3' antisense; TARC, 5'CACG CAGCTCGAGGGACCAARGTG3' sense and 5'TCAAGACCTCT CAAGGCTTTGCAGG3' antisense. PCR conditions were: 94°C for 10 min, 40 cycles of 94°C, 30 s; 59°C, 30 s; 72°C, 30 s; and 5 min at 72°C.

### Measurement of TARC Protein Levels

TARC levels were detected in cell-free supernatants, using an ELISA kit according to the manufacturer's guidelines (R&D Systems).

### Immunofluorescent Stainings

Cytopins were fixated in PBS-buffered paraformaldehyde (4%) for 60 min, permeabilized in PBS containing 0.2% Triton X-100 for 10 min, and blocked with 10% goat serum in PBS for 60 min. Cytopins were washed three times with PBS and incubated for 60 min with the monoclonal anti-TARC antibody (1:100; R&D Systems) or monoclonal anti-p65 antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), which was detected by incubation with Alexa green 488-labeled anti-goat IgG conjugate (1:400) or Alexa green 488-labeled anti-rabbit IgG conjugate (1:400) (Molecular Probes, Eugene, OR). Cytopins were washed with PBS for 15 min and nuclei were stained using DAPI in vectashield (Vector Laboratories, Burlingame, CA). Fluorescence was analyzed by fluorescence microscopy (Zeiss, Gottingen, Germany).

### Migration Assay

The migration assay was performed by using a microchamber transwell system with 5-µm pores (Corning Costar, Corning, NY) as described previously (33). Supernatant derived from Der p-activated and Der p/IL-4/TGF-β-activated 16HBE cells was treated with or without 10 µg/ml neutralizing α-TARC antibody (R&D Systems) for 1 h at 37°C, based on manufacturer's suggestions. Migration was induced by the presence of 300 µl of supernatant in 300 µl RPMI 1640/2% FCS or 0.1–100 ng/ml TARC (R&D Systems) in the lower compartment of the chamber. T cells were allowed to migrate to the lower compartment for 2 h at 37°C.

### Flow Cytometry

Nonmigrating T cells and T cells migrated toward the 16HBE-derived supernatant were analyzed using α-CCR4-PE (Pharmacia, Uppsala, Sweden). An irrelevant specificity antibody of the same isotype was used for gate setting. Analysis was performed using an Elite flow cytometer (Beckman Coulter, Hialeah, FL).

### Immunodetection by Western Blotting

Total cell lysates were obtained by resuspension of the cells in 1× Sample buffer (containing 2% SDS, 10% glycerol, 2% β-mercaptoethanol, 60 mM Tris-Cl pH 6.8, and bromophenol blue) and boiling for 5 min. Phosphorylation of p38, extracellular signal-regulated kinase (ERK) and the 1,173 tyrosine residue of EGFR were analyzed by Western blotting. Samples were loaded on a SDS 10% PAGE gel (acrylamide:bisacrylamide 173:1) and transferred to a PVDF membrane (Millipore, Bedford, MA). Immunodetection of phospho-p38, phospho-ERK (New England Biolabs, Hitchin, Herts, UK), phospho-EGFR, and pan-ERK (Santa Cruz Biotechnology) was performed by standard procedures and the detection was performed according the manufacturer's guidelines (ECL, Amersham, Buckinghamshire, UK). Relative protein levels were quantified using the gelscan program ImageMaster (Pharmacia) and normalized for total protein levels.

### Electrophoretic Mobility Shift Assay

After the cells were harvested, nuclear extracts were prepared by centrifugation at 500 × g for 5 min, washing once with PBS, and resuspension in Buffer A (10 mM HEPES PH 7.9, 10 mM KCl, 0.1 M EDTA, 0.1 mM EGTA, 1 mM DTT) supplemented with protease inhibitors (Complete; Roche, Basel, Switzerland), 1 mM DTT, 1 mM PMSF, 1 mM sodium orthovanadate, 1 µg/ml aprotinin, and 1 µg/ml leupeptin. After 15 min of incubation on ice, cells were lysed by adding Nonidet P-40 (25 µl). Cell lysates were centrifuged at maximal speed for 1 min at 4°C, and nuclear pellets were resuspended in buffer C (20 mM

HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) with protease inhibitors (Complete; Roche), 1 mM DTT, 1 mM PMSF, 1 mM sodium orthovanadate, 1  $\mu$ g/ml aprotinin, and 1  $\mu$ g/ml leupeptin. The suspension was incubated on ice for 20 min with regular vortexing to extract the nuclear proteins. Finally pellets were spun down at maximum speed for 30" and the supernatants containing nuclear extracts were stored at  $-80^{\circ}\text{C}$ . The concentration of the nuclear proteins was determined using the Bradford assay. A fluorescence-labeled double stranded oligonucleotide probe (5'AACAAAGAGGATTTACCTACAT3'-IR dye 700) containing the NF- $\kappa$ B-binding sequence was used in the gel retardation assay. Annealing of the two strands was performed by heating for 2 min at  $95^{\circ}\text{C}$  and slowly cooling down to room temperature. Next, 5  $\mu$ g of nuclear extract and 0.1 ng double-stranded labeled oligo were incubated in 20 mM HEPES (pH 7.9), 60 mM KCl, 0.06 mM EDTA, 0.6 mM DTT, 2 mM spermidine, and 10% glycerol, supplemented with 2  $\mu$ g poly dI-dC) at  $26^{\circ}\text{C}$  for 30 min. The samples were loaded on pre-run (30 min, 100 V) 4% polyacrylamide gels containing 2.5% glycerol and run for 1.5 h at 150 V in  $1.0\times$  tris boric acid EDTA (TBE) at room temperature. The binding signal was quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

### Transfection with the NF- $\kappa$ B Super Repressor Construct

16HBE cells were seeded at 100,000 cells/well and grown overnight in EMEM containing 10% FCS. A construct containing a mutant I $\kappa$ B protein with serine to alanine substitutions at amino acid residues 32 and 36 (pMSCV-HA-I $\kappa$ Balpha-SS32/36AA), which cannot be degraded and therefore acts as potent super repressor of NF- $\kappa$ B activity (a kind gift from Drs. H. Schepers, University Medical Center of Groningen, Groningen, The Netherlands), was constructed after ligation of the  $\pm$  1-kb *Bam*HI fragment from pCDNA3 HA-I $\kappa$ Balpha SS32/36AA into the *Bgl*II site of pMSCV-iGFP (kindly provided by Prof. dr. S.E. Shoelson, Joslin Diabetes Center, Boston, MA, and Dr. J. J. Schuringa, University Medical Center of Groningen, respectively). Cells were transfected with the construct containing the mutant I $\kappa$ B protein or with an empty vector, using 3  $\mu$ l/ml Fugene-6-Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN). When the cells were grown to confluence after 3 d, the medium was replaced by EMEM containing 0.5% FCS. The next day, cells were stimulated for 6 h and mRNA was isolated for further examination.

### Statistical Analysis

We assumed normal distribution and used the Student's *t* test for paired observations to test for significance in all analyses.

## RESULTS

### Regulation of TARC Expression Is Induced by Der p, IL-4, and TGF- $\beta$

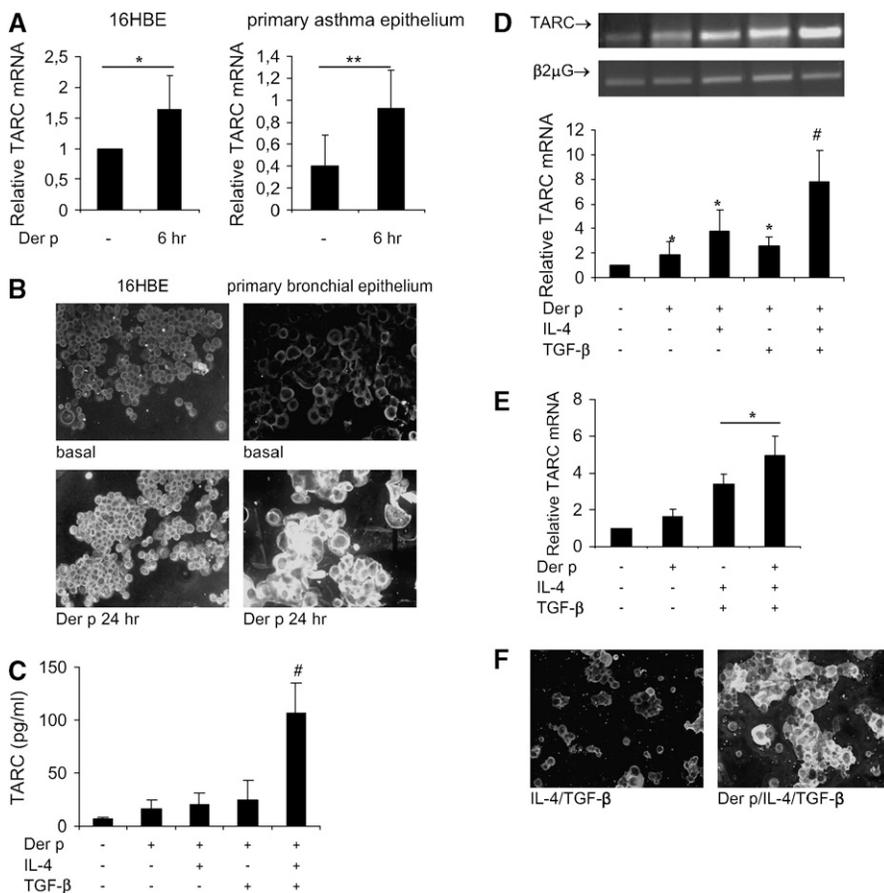
When cells of the bronchial epithelial cell line 16HBE were stimulated with Der p for 6 h, TARC mRNA levels were significantly increased (by  $1.6 \pm 0.4$ -fold, Figure 1A,  $P < 0.05$ ,  $n = 10$ ) when compared with cells that were left unstimulated. In accordance with mRNA levels, protein expression of TARC was up-regulated upon Der p stimulation. In cytopins stained with polyclonal antibody against TARC, we observed only weak immunoreactivity under basal conditions, but a marked increase in TARC protein expression after exposure to Der p for 24 h (Figure 1B). To further examine if Der p-activated bronchial epithelial cells can serve as source of TARC upon allergen challenge, we tested the expression of TARC in primary bronchial epithelial cells from patients with asthma. Similar effects were observed in primary bronchial epithelial cells from patients with asthma. When these cells were stimulated with Der p for 6 h, TARC mRNA levels were significantly increased (by  $1.9 \pm 0.6$ -fold, Figure 1A,  $P < 0.001$ ,  $n = 9$ ). Furthermore, we observed clear TARC immunofluorescence in primary epithelial cells under basal conditions, which was further increased upon cells 24 h stimulation with Der p (Figure 1B). However, in both

16HBE cells (Figure 1C) and the primary asthma epithelium (data not shown), levels of TARC protein in supernatant were often below ELISA standard values ( $< 31$  pg/ml) after stimulation with Der p. To test whether bronchial epithelial cells do secrete TARC upon Der p stimulation, we studied if supernatants from 16HBE cells stimulated with Der p for 24 h were able to induce migration of peripheral blood T cells. A  $2.2 \pm 0.4$  ( $n = 6$ )-fold increase in T cell migration was observed over spontaneous T cell migration (i.e., in the presence of medium alone) when supernatant derived from Der p-stimulated 16HBE cells was present (Figure 2A). Der p itself had no effect on T cell migration (data not shown). To examine the involvement of TARC in this migratory response, we pretreated the supernatants with neutralizing  $\alpha$ -TARC antibody for 1 h at a concentration of 10  $\mu$ g/ml. The antibody had no effect on spontaneous T cell migration, but reduced Der p-induced T cell migration by  $53 \pm 6\%$  ( $P = 0.03$ ,  $n = 6$ ), indicating that biologically active TARC is secreted (Figure 2A). We also tested for the presence of the TARC receptor CCR4 on T migrated cells. A higher percentage of CCR4<sup>+</sup> T cells was observed in the T cell pool that migrated toward the supernatant of Der p-activated 16HBE cells than in nonmigrated T cells from the same donor (Figure 2B). This supports a role for CCR4 ligands in the attraction of T cells to supernatant of Der p-activated bronchial epithelial cells. The dose-dependent T cell migration as induced by rhTARC (in a range of 100 pg to 500 ng/ml) is depicted in Figure 1A.

Given their expression in the asthmatic airways and expression of their receptors on bronchial epithelial cells, we were interested in the effects of Th2-type cytokine IL-4 and TGF- $\beta$ . Interestingly and in contrast to stimulation with Der p alone, TARC protein levels were detectable in the supernatant upon 24 h of co-stimulation with IL-4 and TGF- $\beta$ . A  $9.4 \pm 3.4$ -fold increase ( $n = 4$ ) in TARC secretion was observed when compared with stimulation with Der p alone. In addition, supernatant of 16HBE cells stimulated with Der p, IL-4, and TGF- $\beta$  gave rise to a  $3.1 \pm 0.9$ -fold induction in T cell migration, which was again significantly inhibited by  $\alpha$ -TARC for  $\sim 40\%$  (data not shown). Addition of IL-4 or TGF- $\beta$  alone (fold induction  $2.4 \pm 1.8$  and  $1.9 \pm 0.9$ , respectively,  $n = 3$ ) had a less pronounced effect than addition of their combination, and only the combination of the three stimuli induced a significant increase in TARC levels (Figure 1C). This cooperative effect of IL-4 and TGF- $\beta$  on Der p-induced TARC expression was also observed at mRNA level (with a  $5.7 \pm 3.4$ -fold increase,  $n = 10$ ,  $P < 0.01$ , Figure 1D). Vice versa, Der p increased IL-4- and TGF- $\beta$ -induced TARC mRNA and protein expression (Figures 1E and 1F). These data indicate that the cooperative effect of Der p, IL-4, and TGF- $\beta$  on TARC expression is largely regulated at transcriptional level.

### Signaling Pathways Induced by Der p, IL-4, and TGF- $\beta$ Stimulation

Next, we aimed to define the signaling pathways involved in Der p-, IL-4-, and TGF- $\beta$ -induced TARC production. Der p has been described to activate protease-activated receptor (PAR)-2 (34), a G protein-coupled receptor (GPCR). Activation of GPCR is known to induce release of G $\beta\gamma$  subunits of the G protein, resulting in activation of Src family kinases, recruitment of  $\beta$ -arrestin, and activation of PI3-K. In addition, activation of GPCRs have been described to transactivate the EGFR, possibly through ADAM-dependent shedding of heparin-bound EGFR ligands, which may result in activation of p38 and the MEK-1/ERK-1/2 MAPK pathways (35, 36). Activation of these pathways can induce transcriptional activity of the transcription factor NF- $\kappa$ B (37–39). At present, the intracellular signaling pathways induced by Der p stimulation of epithelial cells are largely unknown. We observed that Der p is able to induce an increase



**Figure 1.** Der p- and cytokine-induced TARC expression in bronchial epithelial cells. (A) 16HBE cells or epithelial cultures from nine different asthma patients were stimulated for 6 h with Der p (50  $\mu$ g/ml) or left unstimulated. TARC expression was determined by real-time PCR and related to the expression of the housekeeping gene  $\beta_2\mu$ G. Mean relative TARC mRNA levels  $\pm$  SEM of nine independent experiments are shown. \* $P < 0.05$  and \*\* $P < 0.01$  between the indicated values. (B) 16HBE cells or primary bronchial epithelial cells derived from patients with asthma were stimulated for 24 h with Der p (50  $\mu$ g/ml) or left unstimulated. Cytospins were prepared and TARC was detected by immunofluorescent staining. Representatives of three independent experiments in 16HBE and nine epithelial cultures from different patients with asthma are shown. (C) 16HBE cells were stimulated for 24 h with Der p (50  $\mu$ g/ml), in presence and absence of IL-4 (10 ng/ml) and TGF- $\beta$  (2 ng/ml). TARC levels ( $x \pm$  SEM) in cell-free supernatants of at least four independent experiments are shown. # $P < 0.01$  between the indicated value and all other values. (D) 16HBE cells were stimulated for 6 h with Der p (50  $\mu$ g/ml), in the presence and absence of IL-4 (10 ng/ml) and TGF- $\beta$  (2 ng/ml). TARC expression was determined by real-time PCR and related to the expression of  $\beta_2\mu$ G. Mean relative TARC mRNA levels  $\pm$  SEM of at least three independent experiments are shown. \* $P < 0.05$  between the indicated value and the unstimulated condition. # $P < 0.01$  between the indicated value and

other values. The corresponding panel demonstrates TARC and  $\beta_2\mu$ G signals after regular RT-PCR and loading of the amplified product on agarose gel containing ethidium bromide. (E) 16HBE cells were stimulated for 6 h with IL-4 (10 ng/ml) and TGF- $\beta$  (2 ng/ml), in the presence and absence of Der p (50  $\mu$ g/ml). TARC expression was determined by real-time PCR and related to the expression of  $\beta_2\mu$ G. Mean relative TARC mRNA levels  $\pm$  SEM of at least three independent experiments are shown. \* $P < 0.05$  between the indicated values. (F) Primary bronchial epithelial cells were stimulated for 24 h with IL-4 (10 ng/ml) and TGF- $\beta$  (2 ng/ml), in the presence and absence of Der p (50  $\mu$ g/ml). Cytospins were prepared and TARC was detected by immunofluorescent staining. Representatives are shown.

in phosphorylation of the EGFR. This effect was dependent on ADAM activity, since ADAM inhibitor TAPI-2 reduced the Der p-induced phosphorylation of EGFR (Figure 3A). In addition, Der p induced phosphorylation of p38 and ERK-1/2, with the most pronounced effect after 5 min of exposure to Der p. This effect of Der p was also dependent on ADAM activity, as demonstrated by the blocking effects of TAPI-2 (Figure 3B). TAPI-2 not only prevented the Der p-induced increase in ERK phosphorylation, but also reduced basal ERK activity, suggesting that there is substantial basal ADAM activity in 16HBE cells. The use EGFR-selective tyrosine kinase inhibitor tyrphostin AG1478 also inhibited basal activity of ERK and blocked the Der p-induced increase of p38 as well as ERK phosphorylation (Figure 3C). These results indicate that the effect of Der p on p38 and ERK activation is mediated by ADAM-dependent phosphorylation of the EGF receptor. Furthermore, Der p induced phosphorylation of Akt, which acts downstream of PI3-K, and Src family kinases. However, in contrast to p38 and ERK, Src kinase and Akt activation was independent on ADAM and EGFR activity (data not shown).

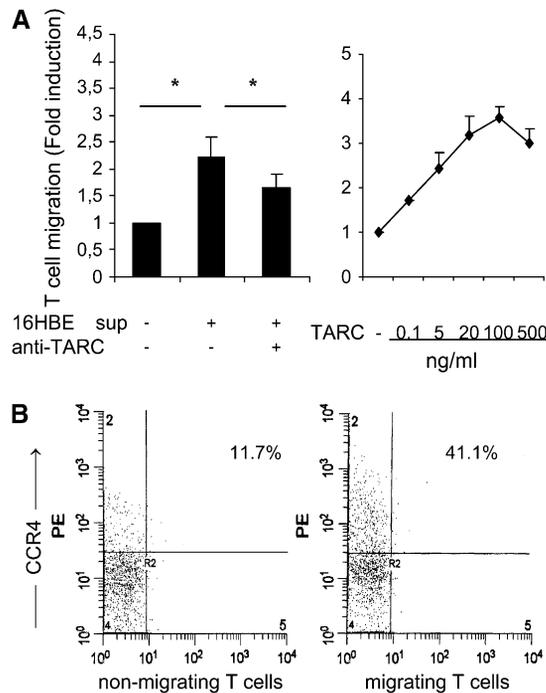
Similar to the effects of Der p in 16HBE cells, we observed an increase in EGFR phosphorylation in primary epithelial cells from patients with asthma. EGFR phosphorylation was again reduced in presence of TAPI-2, indicating that ADAM activity is involved in EGFR activation in primary asthma epithelium

as well (Figure 3D). As expected, phosphorylation of EGFR was also completely blocked by AG1478 (Figure 3E). In addition, Der p increased p38 and ERK phosphorylation in the primary epithelial cells, which could again be inhibited by the ADAM inhibitor, demonstrating that similar pathways are involved in Der p-induced effects as in 16HBE cells (Figure 3F).

In agreement with the data on TARC expression, we observed that the Der p-induced phosphorylation of EGFR cells was enhanced by IL-4 and TGF- $\beta$  in 16HBE cells (Figure 4A), in an ADAM-dependent manner (not shown). Accordingly, presence of IL-4 and TGF- $\beta$  enhanced the phosphorylation of p38 and ERK over stimulation with Der p alone (Figure 4B). The increased phosphorylation of EGFR and downstream activation of p38 and ERK in presence of IL-4 and TGF- $\beta$  suggests that Der p-induced signaling and IL-4- and TGF- $\beta$ -mediated signaling converge at these pathways.

#### Involvement of Signaling Pathways in TARC Expression

We next tested the involvement of the induced EGFR downstream signaling pathways in the expression of TARC. Presence of AG1478 strongly inhibited Der p/IL-4/TGF- $\beta$ -induced TARC mRNA expression ( $P < 0.05$ ,  $n = 3$ ). In addition, we used specific inhibitors of p38 and the MEK-1//ERK-1/2 pathways, SB203580 and U0126, respectively. Der p/IL-4/TGF- $\beta$ -induced transcription of TARC was also significantly inhibited by



**Figure 2.** Supernatant of Der p-stimulated bronchial epithelial cells induces TARC-dependent T cell migration. (A) Supernatant of Der p-stimulated bronchial epithelial cells and increasing concentrations of TARC induce T cell migration. T cells were added to the upper well of a microchamber transwell system. Migration was induced by the presence supernatant or TARC in the lower well, in presence or absence of neutralizing  $\alpha$ -TARC antibody. Migrating T cells were counted, expressed as percentage of the total amount of T cells added to the upper compartment, and related to the spontaneous migration. Experiments were performed in duplicate with T cells from three healthy donors, and means  $\pm$  SEM are shown. \* $P < 0.05$ . (B) CCR4 staining on nonmigrating peripheral blood T cells and T cells that migrated to the lower compartment after 2 h of incubation with the Der p-stimulated 16HBE supernatant. A representative of three independent experiments is shown.

presence of SB203580 and U0126 ( $P < 0.05$ ,  $n = 6$ ), indicating that each of these pathways contributes to TARC expression (Figure 5B). Accordingly, the presence of SB203580 and U0126 inhibited the Der p/IL-4/TGF- $\beta$ -induced protein secretion of TARC (Figure 5B).

#### Involvement of NF- $\kappa$ B in TARC Expression

Activation of MAPK pathways has been described to induce phosphorylation and translocation of the transcription factor NF- $\kappa$ B to the nucleus (37). In addition, NF- $\kappa$ B inhibitors have been reported to diminish TARC expression in the alveolar epithelial cell line A549 (3). To test the possible involvement of NF- $\kappa$ B in the effects of Der p, IL-4, and TGF- $\beta$ , we studied nuclear translocation of the p65 subunit of the active NF- $\kappa$ B complex upon stimulation with Der p alone and in combination with IL-4 and TGF- $\beta$ . After stimulation with Der p, nuclear staining of p65 was increased, which became more evident when IL-4 and TGF- $\beta$  were added as well (Figure 6A). We used SB203580 and U0126 to study involvement of p38 MAPK and ERK-1/2 in the activation of NF- $\kappa$ B. Both SB203580 and U0126 reduced nuclear expression of p65 (Figure 6B).

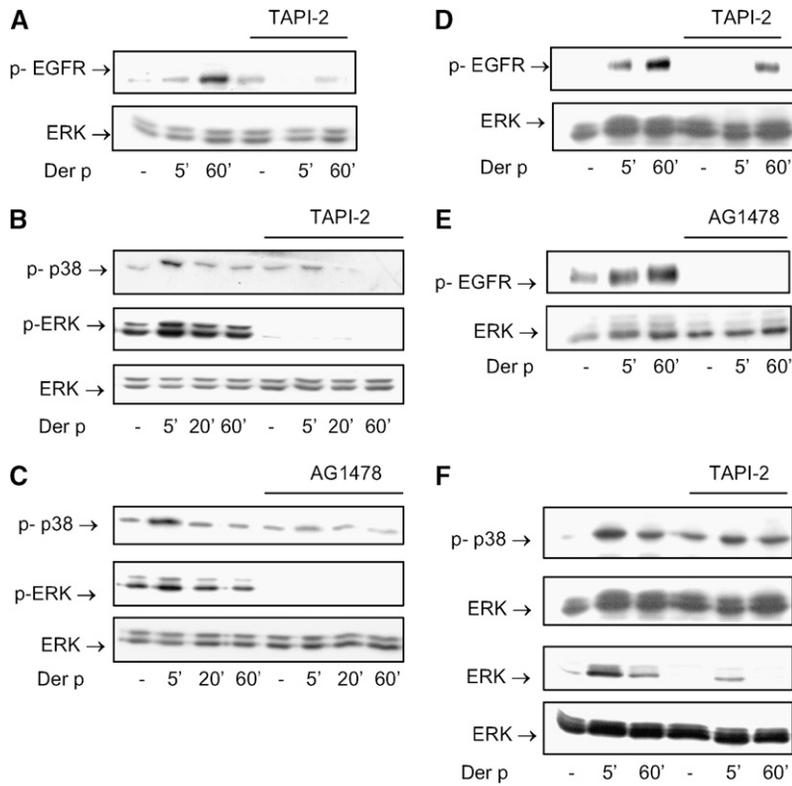
In addition, DNA-binding activity of NF- $\kappa$ B was studied. Der p induced a slight increase in NF- $\kappa$ B binding to its specific sequence, which was a further upregulated when IL-4 and TGF- $\beta$

were added as well. The p38 and ERK inhibitors could again block this effect (Figure 6C). These results indicate that activation of NF- $\kappa$ B may be involved in the cooperative effect of Der p, IL-4, and TGF- $\beta$  on TARC expression. Since binding of NF- $\kappa$ B to its promoter may not necessarily result in transcriptional activation of NF- $\kappa$ B-dependent genes, we confirmed the role of NF- $\kappa$ B in transcriptional regulation of TARC expression. We used a construct with a mutant I $\kappa$ B $\alpha$  protein that does not undergo degradation and therefore acts as super repressor of NF- $\kappa$ B. In cells transfected with the empty control vector, stimulation with Der p or the combination of Der p, IL-4, and TGF- $\beta$  again induced a significant increase in TARC mRNA expression. After transfection with the NF- $\kappa$ B super repressor construct, Der p was no longer able to significantly increase TARC expression, while the effect of Der p in combination with IL-4 and TGF- $\beta$  was significantly inhibited (Figure 6D). This indicates that NF- $\kappa$ B is involved in TARC transcription.

#### DISCUSSION

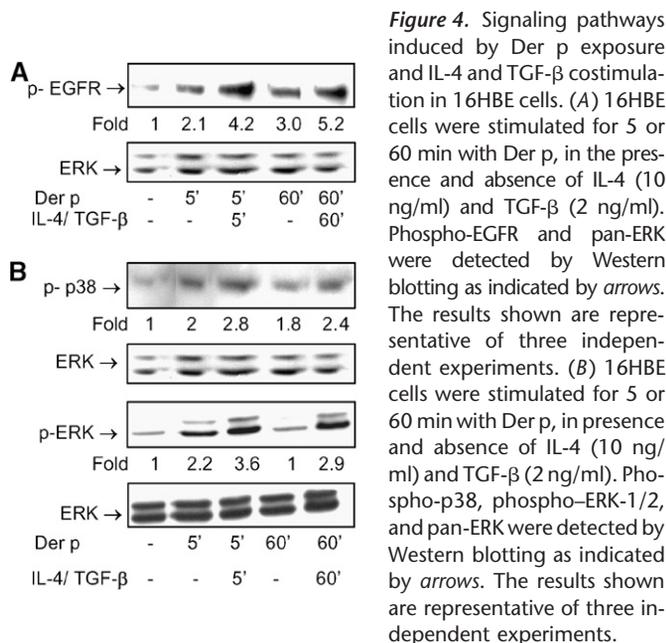
Allergen exposure induces Th2-mediated airway inflammation in sensitized patients with asthma, which is characterized by recruitment of eosinophils and Th2 cells. There is increasing evidence that the chemokine TARC plays a crucial role in the recruitment of Th2 cells during this allergic response. Indeed, an allergen-induced increase in levels of the chemokine TARC has been observed in the airways (2, 6, 40). Upon allergen inhalation, aeroallergens first contact the bronchial epithelium. Although it is known that airway epithelial cells express TARC, it is still largely unclear how the expression of TARC is regulated and whether allergens are able to promote Th2 infiltration directly by induction of TARC expression in bronchial epithelial cells. The present study is the first to demonstrate that Der p allergens from house dust mite induce TARC expression in bronchial epithelial cells. This effect appears to be mediated by ADAM-dependent phosphorylation of EGFR and subsequent activation of MAP kinases and NF- $\kappa$ B. In this way, direct contact of the bronchial epithelium with protease-containing aeroallergens may contribute to the increased TARC expression and recruitment of Th2 cells that has been observed in the asthmatic airways after inhalation of pollen or house dust mite. In addition, contact of the bronchial epithelium with protease containing allergens might contribute to allergic sensitization, since we also observed that Der p is able to up-regulate mRNA expression of Th2 priming factor TSLP (data not shown). TSLP has been shown to induce TARC expression in dendritic cells, and its expression was correlated with TARC expression in bronchial epithelium from patients with asthma (1, 41).

By immunofluorescence we clearly observed TARC protein expression, which was further increased upon Der p stimulation. Instead, levels of TARC protein were hardly detectable in supernatants from Der p-stimulated bronchial epithelial cells. This prompts the question whether TARC may be retained intracellularly upon Der p stimulation. However, supernatants from Der p-stimulated 16HBE cells were able to induce migration of T cells, which was in part dependent on TARC. This suggests that there is secretion of TARC by bronchial epithelial cells and TARC is not merely retained intracellularly. Accordingly, CCR4 expression was increased on T cells that migrated toward Der p-stimulated supernatant when compared with nonmigrated T cells. In consistency with our findings, chemotactic activity of chemokines with concentrations below detection limit has previously been identified in epithelial supernatants (42). After co-stimulation with cytokines characteristic for asthmatic airway inflammation (i.e., Th2-type cytokine IL-4 and the growth factor TGF- $\beta$ ), TARC protein levels were clearly detectable. In



**Figure 3.** Signaling pathways induced by Der p exposure in bronchial epithelial cells. (A) 16HBE cells were stimulated for 5 or 60 min with Der p (50  $\mu\text{g/ml}$ ), in presence and absence of ADAM inhibitor TAPI-2 (22.5  $\mu\text{M}$ ). Total cell lysates were prepared and phospho-EGFR and pan-ERK were detected by Western blotting as indicated by *arrows*. The results shown are representative of three independent experiments. (B) 16HBE cells were stimulated for 5, 20, or 60 min with Der p, in the presence and absence of TAPI-2 (22.5  $\mu\text{M}$ ). Phospho-p38, phospho-ERK-1/2, and pan-ERK were detected by Western blotting as indicated by *arrows*. The results shown are representative of three independent experiments. (C) 16HBE cells were stimulated for 5, 20, or 60 min with Der p, in the presence and absence of tyrosinase AG1478 (1  $\mu\text{M}$ ). Phospho-p38, phospho-ERK-1/2, and pan-ERK were detected by Western blotting as indicated by *arrows*. The results shown are representative of three independent experiments. (D) Primary epithelial cells were stimulated for 5 or 60 min with Der p (50  $\mu\text{g/ml}$ ), in the presence and absence of TAPI-2 (22.5  $\mu\text{M}$ ). Total cell lysates were prepared and phospho-EGFR and pan-ERK were detected by Western blotting as indicated by *arrows*. The results shown are representative of three independent experiments. (E) Primary epithelial cells were stimulated for 5 or 60 min with Der p, in presence and absence of AG1478 (1  $\mu\text{M}$ ). Phospho-EGFR and pan-ERK were detected by Western blotting as indicated by *arrows*. The results shown are representative of three independent experiments. (F) Primary epithelial cells were stimulated for 5 or 60 min with Der p, in the presence and absence of TAPI-2 (22.5  $\mu\text{M}$ ). Phospho-p38, phospho-ERK-1/2, and pan-ERK were detected by Western blotting as indicated by *arrows*. The results shown are representative of three independent experiments.

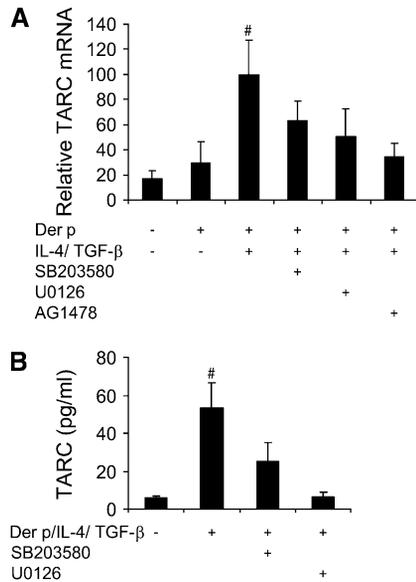
addition, a marked increase in TARC mRNA expression was observed over the presence of Der p alone, indicating that the cooperative effect of IL-4 and TGF- $\beta$  is regulated at transcriptional level. The Der p-induced expression of TARC may thus be promoted by micro-environmental factors in asthmatic airways.



**Figure 4.** Signaling pathways induced by Der p exposure and IL-4 and TGF- $\beta$  costimulation in 16HBE cells. (A) 16HBE cells were stimulated for 5 or 60 min with Der p, in the presence and absence of IL-4 (10 ng/ml) and TGF- $\beta$  (2 ng/ml). Phospho-EGFR and pan-ERK were detected by Western blotting as indicated by *arrows*. The results shown are representative of three independent experiments. (B) 16HBE cells were stimulated for 5 or 60 min with Der p, in presence and absence of IL-4 (10 ng/ml) and TGF- $\beta$  (2 ng/ml). Phospho-p38, phospho-ERK-1/2, and pan-ERK were detected by Western blotting as indicated by *arrows*. The results shown are representative of three independent experiments.

TARC predominantly attracts Th2 cells and IL-4 may act in a positive feedback loop to enhance Th2 cell recruitment to the sites of inflammation. Previously, it has already been demonstrated that IL-4 is able to enhance TNF- $\alpha$ /IFN- $\gamma$ -induced TARC secretion in the airway epithelial cell lines A549 and BEAS-2B (6). In contrast to our findings, an inhibitory effect of TGF- $\beta$  on TARC production has been described in the human keratinocyte cell line HaCaT (43). However, it is important to note that IL-4 also inhibited instead of up-regulated TARC expression in these cells (44) and that inhibition of EGFR tyrosine kinase enhanced instead of inhibited TARC production in these cells (45). TGF- $\beta$  can be produced by stimulation of epithelial cells with Th2 cytokines and is seen as an important cytokine involved in remodeling of the asthmatic airways. Our current data suggest that, by promoting TARC expression in turn, TGF- $\beta$  may also be involved in a positive feedback loop to enhance airway inflammation. In addition to IL-4, the effect of Th2 cytokine IL-13 on TARC expression might be of interest given its persistent effects on airway inflammation and remodeling in asthma (46). Future studies will have to reveal whether IL-13 acts on TARC regulation in a manner similar to that in which it acts on IL-4, although preliminary results have already indicated that IL-13 up-regulates TARC mRNA expression as well (I. H. Heijink and coworkers, unpublished observations, 2006).

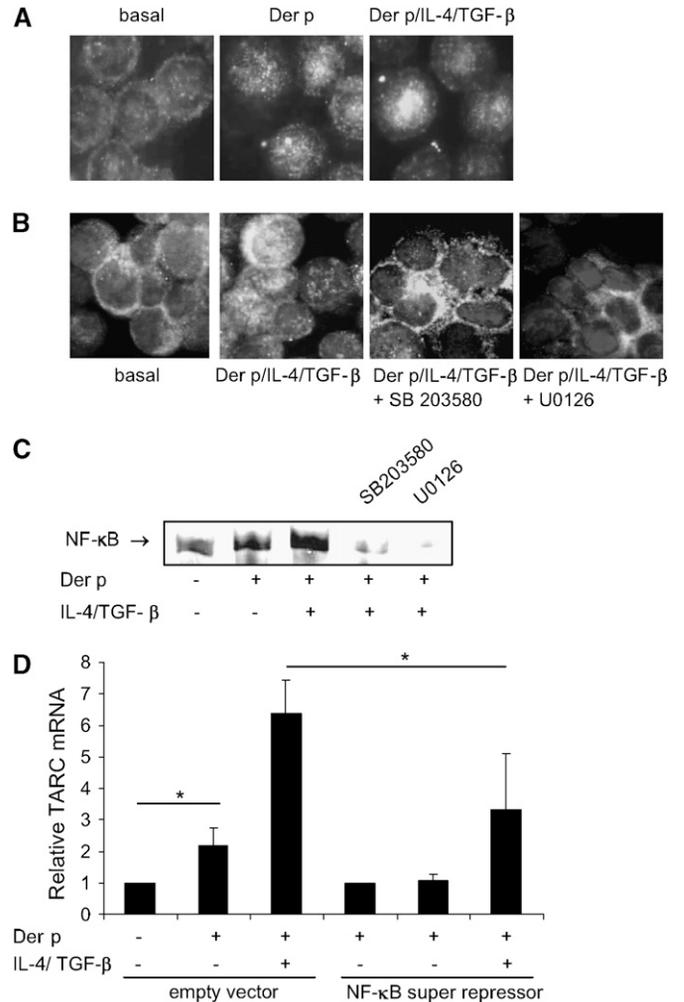
Further studies will elucidate whether IL-4 and TGF- $\beta$  also enhance TARC expression in primary asthma epithelium and may thus be involved in the exaggerated Th2 activity in respiratory allergy. On the other hand, intrinsic factors may render the asthma epithelium more prone to produce proinflammatory mediators (e.g., TARC) and promote Th2-mediated inflammation



**Figure 5.** TARC expression is significantly reduced by selective inhibitors of p38 MAPK, MEK/ERK-1/2, and phospho-tyrosine EGFR. (A) 16HBE cells were stimulated for 6 h with Der p (50  $\mu$ g/ml), in presence and absence of IL-4 (10 ng/ml), TGF- $\beta$  (2 ng/ml), SB203580 (1  $\mu$ M), U0126 (10  $\mu$ M), and tyrphostin AG1478 (1  $\mu$ M). TARC expression was determined by real-time PCR and related to the expression of the house-keeping gene  $\beta_2\mu$ G. Mean relative TARC mRNA levels  $\pm$  SEM of at least three independent experiments are shown. <sup>#</sup> $P < 0.05$  between the indicated value and all other values. (B) 16HBE cells were stimulated for 24 h with Der p (50  $\mu$ g/ml), in presence and absence of IL-4 (10 ng/ml), TGF- $\beta$  (2 ng/ml), SB203580 (1  $\mu$ M), and U0126 (10  $\mu$ M). TARC levels ( $\times \pm$  SEM) in cell-free supernatants of at least four independent experiments are shown. <sup>#</sup> $P < 0.05$  between the indicated values and all other values.

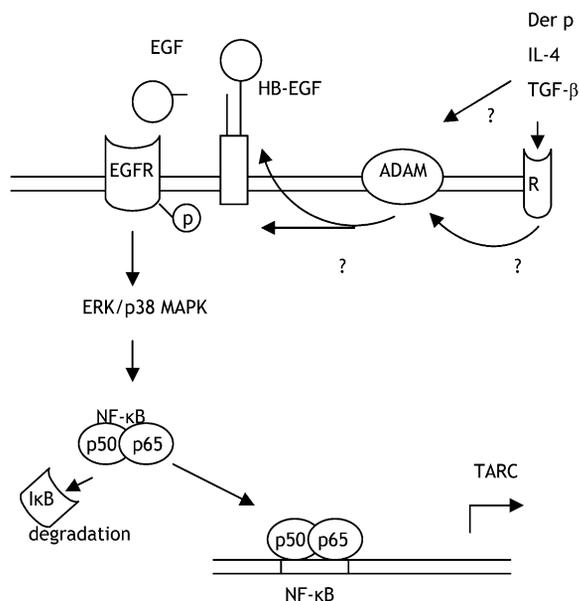
upon allergen exposure. Previously, it has been demonstrated that primary epithelial cell cultures from patients with asthma produce higher levels of the chemokine CCL20 upon stimulation with the cysteine protease Der p 1 than do healthy control subjects (47). This finding suggests that epithelial cells from patients with asthma are more prone to allergens that contain proteases. Indeed, increased PAR-2 expression has been observed in the respiratory epithelium of patients with asthma. This may lead to increased sensitivity to proteases (22, 48, 49). In contrast, another study demonstrated no differences in IL-8 and GM-CSF release upon Der p stimulation in bronchial epithelial cultures from healthy and asthma donors. However, healthy epithelium failed to release TGF- $\alpha$  upon stimulation in this study, while asthma epithelium did (26). The discrepancy between effects on TGF- $\alpha$  and the other cytokines is unresolved, but it might be linked to variations in ADAM activity, since ADAM-17 is involved in the release of TGF- $\alpha$  (36).

The present study is the first to demonstrate that Der p is capable of inducing ADAM-dependent EGFR phosphorylation and subsequent activation of p38 and ERK-1/2 in both 16HBE and primary asthma bronchial epithelial cells (as depicted in Figure 7). It has previously been demonstrated that trypsin-induced PAR-2 activation results in metalloproteinase-dependent cleavage of EGF ligands and EGFR transactivation in colon cancer (35), and other GPCR have been described to activate ADAMs. Although we observed similar effects of trypsin (i.e., phosphorylation of EGFR, p38, and ERK-1/2 MAPK; data not shown) in 16HBE cells, further studies will confirm the involvement



**Figure 6.** NF- $\kappa$ B is activated by Der p, IL-4 and TGF- $\beta$  in 16HBE cells and involved in TARC expression. (A) 16HBE cells were left unstimulated or stimulated for 3 h with Der p (50  $\mu$ g/ml), in the presence and absence of IL-4 (10 ng/ml) and TGF- $\beta$  (2 ng/ml). Cytospins were prepared and p65 was detected by immunofluorescent staining. Representatives of three independent experiments are shown. (B) 16HBE cells were left unstimulated or stimulated for 3 h with Der p (50  $\mu$ g/ml), IL-4 (10 ng/ml), and TGF- $\beta$  (2 ng/ml), in the presence and absence of SB203580 (1  $\mu$ M) and U0126 (10  $\mu$ M). Cytospins were prepared and p65 was detected by immunofluorescent staining. (C) 16HBE cells were stimulated for 3 h with Der p (50  $\mu$ g/ml), in presence and absence of IL-4 (10 ng/ml), TGF- $\beta$  (2 ng/ml), SB203580 (1  $\mu$ M), and U0126 (10  $\mu$ M). Nuclear extracts were prepared and effects on NF- $\kappa$ B DNA binding activity were determined. Representatives of three independent experiments are shown. (D) 16HBE cells were transfected with the NF- $\kappa$ B super repressor  $\kappa$ B-A32/36 construct or the empty vector and stimulated for 6 h with Der p (50  $\mu$ g/ml), in the presence and absence of IL-4 (10 ng/ml) and TGF- $\beta$  (2 ng/ml). TARC expression was determined by real-time PCR and related to the expression of  $\beta_2\mu$ G. Mean relative TARC mRNA levels  $\pm$  SEM are shown ( $n = 3$ ). <sup>\*</sup> $P < 0.05$  between the indicated values.

of PAR-2 in the Der p-induced activation of ADAMs. In addition to the effect of Der p, we show a novel, cooperative effect of IL-4 and TGF- $\beta$  on Der p-induced EGFR phosphorylation, which also appeared dependent on ADAM activity. At present little is known about the pathways involved in ADAM-mediated EGFR transactivation. A role for the MAPK pathways has been described in ectodomain shedding of proteins that are cleaved by



**Figure 7.** Putative pathways involved in Der p-, IL-4-, and TGF- $\beta$ -induced transcriptional activation of the TARC gene. Der p, IL-4, and TGF- $\beta$  induce ADAM-dependent phosphorylation of the EGF receptor (EGFR), leading to activation of the MAPK pathways, release of the active p50 and p65 subunit of NF- $\kappa$ B, binding to the TARC promoter, and induction of transcriptional activation.

ADAMs (50). However, we observed that ERK-1/2 phosphorylation was completely dependent on ADAM activity, rendering it unlikely that this pathway is involved in initial ADAM activation. In addition to MAP kinases, involvement of Src family kinases has been demonstrated in EGFR transactivation (35). However, in our setting the Der p-induced phosphorylation of p38 and ERK could not be inhibited by Src family kinase inhibitor PP2 (data not shown), indicating that activation of Src kinases is not involved in ADAM activation and the downstream effects. Further studies will elucidate the precise molecular mechanism involved in ADAM activation.

Our data indicate that EGFR-dependent signaling pathways regulate TARC gene transcription. The phosphorylation of the EGFR results in p38 and ERK-1/2 MAPK-dependent activation of NF- $\kappa$ B activity, which is maximal upon costimulation with Der p, IL-4, and TGF- $\beta$ . Moreover, repression of NF- $\kappa$ B activity reduced TARC expression, indicating that TARC functions as a target gene of NF- $\kappa$ B. Additional transcription factors may also be involved in the regulation of TARC expression. For instance, it has been shown that TARC expression is dependent on STAT6 in STAT6-deficient mice (51).

Together, our results demonstrate that direct interaction of aeroallergens with bronchial epithelial cells may enhance TARC expression and thus contribute to the recruitment of CCR4<sup>+</sup> T cells to the sites of allergen-induced inflammation. The presence IL-4 and TGF- $\beta$  markedly increases the effects of Der p, suggesting that presence of these cytokines in the airway walls may promote allergen-induced TARC production and Th2-mediated airway inflammation. In addition, the involvement of ADAM-dependent activation of EGFR in the induced signaling pathways and regulation of TARC expression suggests that interference with ADAM activity and EGFR tyrosine phosphorylation may be an important new lead in the search for valuable therapeutic strategies for the treatment of asthma.

**Conflict of Interest Statement:** None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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