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IL-9-Mediated Induction of Eotaxin1/CCL11 in Human Airway Smooth Muscle Cells

Abdelilah Soussi Gounni, Qutayba Hamid, Sahidur M. Rahman, Jutta Hoeck, Jie Yang and Lianyu Shan

Recent work has shown the potential importance of IL-9 in allergic diseases. The development of transgenic mice overexpressing IL-9 has suggested a key role for this cytokine in the development of the asthmatic phenotype including airway eosinophilia. In this study, we evaluated the expression of the IL-9R and the effects of IL-9 on human ASM cells by examining the release of Th2-associated chemokines (eotaxin1/CCL11 and thymus- and activation-regulated chemokine (TARC)/CCL17). IL-9R α-chain mRNA and surface expression were detected in cultured human airway smooth muscle (ASM) cells. In addition, primary cultured ASM cells, as well as bronchial smooth muscle cells within biopsies of asthmatics and not control subjects, revealed IL-9R protein expression. IL-9 stimulation of human ASM cells resulted in release of eotaxin1/CCL11, but had no effect on TARC/CCL17. Treatment with Act D abrogates IL-9-induced significantly IL-9-induced production of eotaxin1/CCL11 from ASM cells. Interestingly, real-time RT-PCR showed that IL-9 up-regulated eotaxin1/CCL11 mRNA expression, but had no effect on TARC/CCL17. Treatment with Act D abrogates IL-9-induced eotaxin1/CCL11 mRNA and protein release by ASM cells. Finally, transfection study using eotaxin1/CCL11 promoter luciferase construct confirmed that IL-9 induced eotaxin1/CCL11 at the transcriptional level. Taken together, these data provide new evidence demonstrating that IL-9-dependent activation of ASM cells contributes to eosinophilic inflammation observed in asthma. The Journal of Immunology, 2004, 173: 2771–2779.

Asthma is a chronic disease of the airways characterized by reversible airway obstruction and airway hyperresponsiveness. The histopathology of asthmatic airways shows infiltration of a large number of inflammatory cells, a marked increase in the smooth muscle layer, and significant desquamation of the epithelium (1, 2). Recently, a number of studies have revealed that airway smooth muscle (ASM) cell division and growth can be modulated by diverse factors, including cytokines (3, 4), and therefore, may be linked to the observed hyperplasia and hypertrophy of smooth muscle in asthmatic airways (5). In addition to their proliferative and contractile properties, studies have shown that cultured ASM cells may express chemokines and cytokines (6), thereby acting as effector cells in initiating or perpetuating airway inflammation.

IL-9 is a T cell-derived cytokine with pleiotropic activities on various cell types (7–9). The expression of IL-9 is detectable mainly in activated CD4+ T cells (10). It has been shown that IL-9 can promote the proliferation of activated and transformed T cells, the production of immunoglobulins by B cells, the proliferation and differentiation of mast cells, and erythroid progenitors (9). Recently, a number of observations have suggested that this cytokine may play a role in asthma (8, 9). In humans, the IL-9 gene is located on chromosome 5q31-q33, in a region where a linkage with asthma and its risk factors has been demonstrated (11, 12). More recently, the development of transgenic mice overexpressing IL-9 has suggested a potential role for this cytokine in the development of airway eosinophilia, mast cell hyperplasia, mucus production, and airway hyperresponsiveness (13–15). Like all cytokines, IL-9 functions through cognate interactions with its own distinct receptor, consisting of a ligand-specific α subunit (IL-9R α-chain (IL-9Rα)) and a common γ-chain, which is shared with IL-2, IL-7, and IL-15 (9). Interestingly, a linkage for asthma and broncho-hyperresponsiveness has been also reported with the long arm pseudo-autosomal regions of the X and Y chromosomes, where the IL-9R α gene is located (16). Collectively, these findings support the concept that IL-9 may significantly be involved in mediating both airway inflammation and hyperresponsiveness responsiveness that characterizes the asthmatic state.

With the increasing evidence of structural cell involvement in airway diseases, we hypothesize that ASM cells express IL-9R, and that IL-9 produced by infiltrating inflammatory cells, mainly CD4+ T cells, may affect ASM cell function. In this report, we showed that human ASM express both IL-9Rα mRNA and membrane-bound surface receptor. Immunofluorescence study revealed that ASM cells within bronchial biopsies of asthmatic subjects and cultured primary ASM cells express IL-9Rα. Furthermore, stimulation of ASM cells with IL-9 alone induces eotaxin1/CCL11 release, but not thymus- and activation-regulated chemokine (TARC)/CCL17, in a time- and dose-dependent manner. Neutralizing anti-IL-9 mAb specifically blocked IL-9-mediated eotaxin-1 release from ASM cells.
However, neither anti-IL-4 nor IL-13-neutralizing Ab has an effect, suggesting that IL-9 induction of eotaxin1/CC11 in ASM cells is not mediated through IL-4 or IL-13 pathway. In vitro chemotaxis assay demonstrated that conditioned medium (CM) from IL-9-stimulated ASM cells attracted human eosinophils. Moreover, using the inhibitor of transcription actinomycin D (Act D), transient transfection, and real-time RT-PCR, we showed that IL-9 induces eotaxin1/CC11 expression in primary ASM cells at the transcriptional level. Our results support the idea that human ASM cells can participate in the chemotraction of inflammatory cells, particularly eosinophils, via an IL-9-dependent pathway.

Materials and Methods

Reagents and Abs

Mouse mAb anti-human IL-9Rα directed to N-terminal extracellular domain, recombinant human IL-9, IL-13, IL-4, IL-1β, neutralizing goat anti-human IL-13, and anti-human IL-4 are from R&D Systems (Minneapolis, MN). Neutralizing mouse IgG1 anti-human IL-9 mAb was from Biolegend (San Diego, CA). Mouse IgG1 isotype control (clone MOPC21), and goat IgG were from Sigma-Aldrich (Oakville, Ontario, Canada). FITC-conjugated rat anti-mouse IgG was from Jackson Immunoresearch Laboratories (West Grove, PA), Goat anti-mouse IgG F(ab')2 AlexaFluor 488 was from Molecular Probes (Eugene, Oregon). FBS was from HyClone (Logan, UT). RPMI 1640 medium (penicillin, streptomycin, dNTP, SuperScript reverse transcriptase, and Taq polymerase were from Invitrogen Life Technologies, Grand Island, NY). Unless stated otherwise, all other reagents were obtained from Sigma-Aldrich.

Preparation of human bronchial ASM cells

Human ASM cells (passage 2–5) were obtained from main bronchial airway segments (0.5–1.0 cm diameter) in pathologically uninvolved segments of resected lung specimens using isolation methods described previously (17, 18). Briefly, the muscle layer from each bronchial segment was dissected free from adventitia and submucosa under a binocular dissection microscope, then was minced, and cells were dissociated enzymatically (600 U/ml collagenase I, 10 U/ml elastase (Invitrogen Life Technologies), 2 μl Nagarse protease) for up to 60 min. Cells were seeded at a density of 8000 cells/cm² and grown at 37°C in DMEM supplemented with 10% FBS, sodium pyruvate (1 mM), t-glutamine (2 mM), nonessential amino acid mixture (1:100), A gentamicin (50 μg/ml), and aminopterin B (1.5 μg/ml). Media was replaced every 2 days, and confluent cultures were passaged and reseeded using a split ratio of 1:4. When necessary, cells were stored at −80°C in 10% DMSO/90% FBS. At confluence, primary human ASM cells exhibited spindle morphology and a hill-and-valley pattern that is characteristic of smooth muscle in culture. Moreover, using cultures up to passage 5, over 90% of the cells at confluence retain smooth muscle-specific actin, SM22, and calponin protein expression, and mobilize intracellular Ca²⁺ in response to acetylcholine, a physiologically relevant contractile agonist (17). The growth rate (determined by cell number) of the human ASM cells from all lung resection donors was similar to what has been reported previously for ASM cultures from healthy human transplant donors (17, 18). Morphologically, ASM cells from different lung resection donors and from healthy human transplant donors were indistinguishable.

Study population

Asthmatic patients were recruited from the asthma clinic (Chest Hospital, Montreal, Quebec, Canada) and their characteristics were previously described (19). Ten patients with positive skin tests to at least one aeroallergen and diagnosis of asthma, as defined by the American Thoracic Society, were studied (20). Written informed consent was obtained, and the appropriate institutional review board approved the study. None of the subjects was a current smoker and all had less than a five packyear history of smoking. Patients who had not received inhaled or systemic corticosteroids in the last 3 mo and were not receiving medications other than inhaled β agonists. Patients had not suffered symptoms of an upper respiratory tract infection within the past month. Nebulized salbutamol was given to all subjects before bronchoscopy. Endobronchial biopsies were obtained as previously described (21, 22).

Cell line and culture conditions

The human cell line HL-60 was provided from American Type Culture Collection (Manassas, VA). Cells were cultured at 37°C in humidified 5% CO₂ in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Differentiation toward eosinophils was performed as described previously (23).

RNA isolation and RT-PCR

Total cellular RNA was extracted from serum-fed cultured human ASM cells or HL-60 cell line using TRizol method (Invitrogen Life Technologies, Grand Island, NY). Reverse transcription was performed by using 2 μg of total RNA in a first-strand cDNA synthesis reaction with SuperScript reverse transcriptase as recommended by the supplier (Invitrogen Life Technologies). PCR was performed by adding 1 μl of the reverse transcription product into 50 μl of total volume reaction containing 1× buffer, 200 μmol of each dNTPs, 20 pmol of each oligonucleotide primer, and 0.2 unit of Taq polymerase. Oligonucleotides specific for IL-9R sequences were used in the PCR. Oligonucleotide primers were synthesized on the basis of the entire coding region of the human IL-9Rα (GenBank accession no. M84747) as follows: 5’ primer, 5’-GCAAATCAGTTCTG GCCAC-3’; and 3’ primer, 5’-TGTTCCAGGTCCCGA-3’. The PCR (IL-9Rα, 35 cycles; β-actin, 23 cycles) was conducted in a thermal cycler (PTC100; MJ Research, Watertown, Mass). Each cycle including denaturation (94°C, 1 min), annealing IL-9Rα (60°C, 1 min), β-actin (55°C, 2 min), and extension (72°C, 1 min 30 s). The initial denaturation period was 5 min and the final extension was 10 min. β-Actin was amplified as internal control. Amplified products were analyzed by DNA gel electrophoresis in 2% agarose, and visualized by ethidium bromide staining under UV illumination. The specificity of the amplified band was confirmed by sequencing (data not shown).

Flow cytometry analysis of IL-9R expression

Confluent serum-fed adherent cells were washed with HBSS, detached, and trypsinized (3 min at 37°C) and then washed with HBSS/5% FBS, centrifuged, and transferred to FACS tube. Cells were incubated for 1 h on ice with mAb anti-IL-9Rα or IgG1 isotype control (both at 20 μg/ml). The cells were washed twice with HBSS/2% FBS and incubated with FITC-conjugated rat anti-mouse IgG (1:200) in dark for 30 min on ice. The cells were washed again with HBSS/0.2% FBS, fixed for 10 min in 2% paraformaldehyde, and analyzed on FACSscan. The results are presented as specific mean fluorescence intensity using CellQuest software (BD Biosciences, Oxnard, CA).

Immunofluorescence

Serum-fed human ASM cells grown on 8-well glass slides (Nunc, Naperville, IL) were cultured up to semiconfluence. Slides were fixed with 4% paraformaldehyde, air dried, and stored at −20°C until use. Fresh-frozen sections (5 μm) or 8-well glass slides were saturated with universal blocking solution for 10 min (DakoCytomation, Carpinteria, CA). Slides were incubated with 10 μg/ml anti-IL-9Rα mAb or isotype-matched control (MOPC21) in dilution buffer (DakoCytomation) overnight at 4°C, washed twice with TBS followed by incubation for 1 h at room temperature with goat anti-mouse IgG (F(ab')2, AlexaFluor 488 (1/100 dilution) (Molecular Probes). Slides were then extensively washed with TBS and counterstained with nuclei stain DAPI for 2 min. After washing with PBS, the slides were then mounted with anti-fade agent (Molecular Probes). Samples were photographed on Olympus AX70 microscope with a Photometrics PXL-cooled CCD Camera (Tucson, AZ) and Image-Pro Plus Software (Cargen Group, Markham, Ontario, Canada). The relative expression of IL-9R within ASM cells area of asthmatics and normal control samples were determined by using National Institutes of Health processing Image program (ImageJ 1.31V, written by W. Rasband, National Institutes of Health, Bethesda, MD) as previously described (24).

Cell stimulation and chemokine release assay

Confluent human ASM cells (passage 2–5) were grown arrested by FBS deprivation for 48 h in Ham’s F-12 medium containing 5 μg/ml human recombinant insulin, 5 μg/ml human transferrin, 5 ng/ml selenium, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were then stimulated in fresh FBS-free medium containing graded concentration (0.1, 1, 10, and 100 ng/ml) of human rIL-9, IL-1β, or medium alone. Supernatants were collected at different time points (12, 24, 36, 48, and 72 h), centrifuged at 1200 rpm for 7 min at 4°C to remove cellular debris, and stored at −80°C until analysis by ELISA. For neutralizing experiments, goat anti-IL-13 or anti-IL-4, mouse anti-IL-9-neutralizing Abs were added for 1 h to serum-deprived ASM cells before stimulation with IL-9, IL-13, or IL-4 (10 ng/ml) for 24 h. Goat IgG control or mouse IgG1 control both at 1 μg/ml were used as negative control. Supernatants were then harvested at 24 h and processed as described above.
ELISA analysis of chemokine protein release in cell supernatants

Immunoreactive eotaxin 1/CCL11 and TARC/CCL17 within the supernatants was quantified using ELISA with matched Abs according to protocol previously described (25, 26). Recombinant human chemokines, anti-chemokine capture mAbs, and biotinylated anti-chemokine detection mAbs were obtained from R&D Systems. Chemokine protein was quantified in reference to serial dilutions of recombinant standards falling within the linear part of the standard curve for each specific chemokine measured. The sensitivities of the eotaxin1/CCL11 and TARC/CCL17 assays were 7.8 and 3.9 pg/ml, respectively. Each data point represents readings from a minimum of three independent assays performed in triplicate.

In vitro chemotaxis assay

Human eosinophil purification was performed as we described previously (23). Briefly, blood was collected into sterile heparinized syringes from the peripheral veins of normal donors following informed consent. The erythrocytes were sedimented with dextran, the granulocyte fraction was obtained by centrifugation through a cushion of Ficoll-Hypaque (1.077g/ml; Pharmacia, Uppsala, Sweden) of the buffy coat at 350 g for 30 min. After the hypotonic lysis of residual erythrocytes, eosinophils were separated from neutrophils by anti-CD16 mAb bound to immunomagnetic beads (negative selection) with auto MACS column (Miltenyi Biotec, Auburn, CA). Cyto centrifugation slides of the eosinophils stained with Wright’s Giemsa showed that the purity of the isolated eosinophils was >97%. Chemotaxis experiments were performed in a 24-well microchemotaxis chamber (Corning Glass, Corning, NY). Chemotactic properties of ASM cells were measured by evaluating the chemotaxis migration through a 5-μm polycarbonate filter. The cells were suspended at 1 × 10^5 cells/ml in complete DMEM plus 0.5% BSA, and were added to the top chamber. Various dilutions of IL-9-stimulated ASM cells CM (1/5, 1/10) were added to the bottom chamber. After 2 h of incubation at 37°C with 5% CO2, cells transmigrated into the lower chamber were recovered by centrifugation and counted using automatic cell counter (Guava Technologies, Hayward, CA).

Real-time RT-PCR analysis

Confluent human ASM cells (passage 2–5) were grown arrested by FBS deprivation for 48 h in Ham’s F-12 medium containing 5 μg/ml human recombinant insulin, 5 μg/ml human transferrin, 5 ng/ml selenium, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The cells were pretreated or not with Act D (5 μg/ml) for 1 h and then stimulated in fresh FBS-free medium containing human IL-9, IL-1β (both at 10 ng/ml), or vehicle (PBS) for 6 and 24 h. Cells were then harvested and RNA purified using guanidinium isothiocyanate method (27). Relative levels of eotaxin1/CCL11 and TARC/CCL17 mRNA were analyzed using semiquantitative real-time PCR analysis using LightCycler (Roche, Basel, Switzerland). Predeveloped primers for detection of human eotaxin1/CCL11 and TARC/CCL17 mRNA were purchased from LC-Search (Heidelberg, Germany) and standard manufacturer’s protocol. Briefly, the amplification of target genes in stimulated cells was calculated by first normalizing to the amplification of GAPDH, and then expressing the normalized values as fold increase over the value obtained with unstimulated control cells.

Eotaxin promoter luciferase reporter constructs and cell transfection

Eotaxin promoter was amplified from human cell genomic DNA using PCR with specific primers as described previously (26, 29). Briefly, the amplified fragment 2.25 kb was inserted into pGL3-Basic (Promega, Madison, WI) yielding the reporter construct pGL3-EO2. Constructs used for transient transfection were purified by cesium chloride density gradients. ASM cells (4 × 10^6) were seeded into 12-well plates in complete medium 10% FBS/DMEM. At 50% confluency, transfection was performed in triplicate with 1 μg DNA containing liposomes using Effectene according to manufacturer’s instructions (Qiagen, Valencia, CA). In each well, eotaxin promoter luciferase DNA (pGL3-EO2, 1 μg per sample) and Renilla luciferase reporter vectors (pRL-TK 0.2 μg per sample) were cotransfected and incubated for 24 h. The medium was changed and cells were then stimulated with 10 ng/ml IL-9, IL-β, or IL-4, and then harvested at 12 h. Cells were also stimulated with IL-4 (10 ng/ml) as positive control. The luciferase activity was measured using a luminometer (model LB9501, Berthold Lu-

mat, EGG Berthold, Gaithersburg, MD) and a Dual Luciferase Reporter Assay System (Promega). Briefly, 20 μl of cell lysate was mixed with 100 μl of Luciferase Assay Reagent II (Promega) and firefly luciferase activity was first recorded. Then, 100 μl of Stop-and-Glo Reagent (Promega) was added, and Renilla luciferase activity was measured. All values were normalized to Renilla luciferase activity and expressed relatively to the control transfected nonstimulated cells.

Statistical analysis

Data were obtained from experiments performed in triplicate and repeated at least three times, and results are expressed as geometric mean ± SD. Statistical significance was determined using a Mann-Whitney U test, and p values <0.05 were considered statistically significant.

Results

IL-9Rα is expressed on the surface of human ASM cells

To investigate whether the IL-9Rα is expressed on the cell surface of human ASM cells, we performed flow cytometry on single-cell suspensions of human ASM cells from five different donors using mAb against human IL-9Rα mAb. Analysis of these samples revealed substantial specific fluorescence on the surface of subpopulations of human ASM cells (MFI 118 ± 27.4, n = 5; Fig. 1A). Furthermore, a specific signal was also detected in primary human neutrophil- and eosinophil-differentiated HL-60 cell line used as positive control (Fig. 1, B and C).

mRNA expression of IL-9Rα in human ASM cells

Then, we determined whether human ASM cells express steady-state IL-9Rα mRNA levels. RNA preparation from human ASM cells was first analyzed by RT-PCR. As shown in Fig. 2 (upper panel), mRNA of IL-9Rα was detected in all RNA preparations from four different human ASM cells as well as from eosinophil-differentiated HL-60 cell line. The presence of IL-9Rα mRNA in human ASM cells was confirmed using RT-PCR with other IL-9R-specific primers and sequencing (data not shown). β-Actin-specific amplification products were of similar intensity between all samples, suggesting equality of the RNA preparations (Fig. 2, lower panel).

Expression of IL-9Rα in cultured primary ASM and bronchial smooth muscle cells from asthmatic subjects

To further investigate the protein expression of the IL-9Rα by human ASM cells, immunofluorescence staining was performed with mAb anti-IL-9Rα. A specific staining was observed in cultured human primary ASM cells (Fig. 3A). Substitution of the first Ab with isotype control eliminated the positive signal, demonstrating the specificity of the analysis (Fig. 3B). Then, we examined the expression of IL-9Rα in bronchial ASM cells within biopsies of 10 asthmatic asthmatics and 5 normal donors. As shown in Fig. 3, C–E, specific immunofluorescent signal was clearly observed in smooth muscle cells bundle from an asthmatic subject. However, very weak or no staining was detectable in the smooth muscle area of a normal subject (Fig. 3, D–F). Furthermore, analysis of the relative expression of IL-9Rα in ASM cells area revealed an increase of IL-9Rα expression (intensity of staining or mean Gy value) in asthmatics (mean Gy value ± SD = 85.2 ± 22.9, n = 10) compared with normal control subjects (mean Gy value ± SD = 5.7 ± 3.9, n = 5) (Table I). In both asthmatic and normal control subjects, bronchial epithelial cell layer showed a positive IL-9Rα immunostaining as previously demonstrated (30, 31). Taken together, these results demonstrated that human ASM cells express IL-9R in vitro and in vivo.
IL-9 induces eotaxin1/CCL11 release but not TARC/CCL17 by human ASM cells

Expression of cytokines and chemokines within the airway is a common feature of airway inflammation (2). Moreover, previous studies have clearly demonstrated that IL-9 induces airway inflammation, particularly eosinophilia (13–15, 23, 32). Then, we investigated the effect of IL-9 on eotaxin1/CCL11 and TARC/CCL17. ASM cells were first incubated with graded concentrations of IL-9 or IL-1β/H9252 (0.1, 1, 10, and 100 ng/ml), and the release of eotaxin1/CCL11 and TARC/CCL17 in culture medium was evaluated at different time points (12, 24, 36, 48, and 72 h) by ELISA. Similarly to IL-1β/H9252, IL-9 induced the synthesis and release of eotaxin1/CCL11 in a dose- and time-dependent manner (Fig. 4, A and B). Interestingly, ASM cells stimulated with IL-9 at 1, 10, or 100 ng/ml released significant amounts of eotaxin1/CCL11, compared with unstimulated cells at 24, 48, and 72 h (Fig. 4A). At the early time point (12 h), only 100 ng/ml IL-9 induced significant amount of eotaxin1/CCL11 compared with unstimulated cells. Furthermore, up to 1 ng/ml IL-9 maximum release of eotaxin1/CCL11 is observed by 24 h, and at higher concentrations (10 and 100 ng/ml) mean values of eotaxin1/CCL11 levels increase, although statistically insignificant between 24, 36, 48, and 72 h. Interestingly and in contrast to eotaxin1/CCL11 release, IL-9 was not able to induce TARC/CCL17 on primary ASM cells at any concentration or time point tested (data not shown).

CM from IL-9-stimulated ASM cells promotes in vitro chemotaxis of human eosinophils

Eotaxin1/CCL11 is a well-known potent chemotactic factor for eosinophils (33). We tested the migratory response of human eosinophils toward IL-9-treated ASM cells CM using in vitro chemotaxis assay. Fig. 5 showed that CM from IL-9- or IL-1β-treated ASM cells (1/5) enhanced human eosinophils migration by 2- and 3-fold, respectively, compared with CM from unstimulated ASM cells. This effect is not due to chemokinesis because IL-9-treated ASM cells CM in both upper and lower chambers did not enhance neutrophils migration (data not shown). Moreover, neutralizing mAb but not the isotype control directed against eotaxin1/CCL11 significantly reduced eosinophils migration toward CM from IL-9 ASM cells. In aggregate, these data suggest that IL-9-mediated ASM cells release of eotaxin1/CCL11 participate in eosinophil recruitment.

IL-9-induced eotaxin1/CCL11 on ASM cells is not mediated via IL-13 or IL-4

To gain more insight into the mechanism by which IL-9 induced eotaxin1/CCL11 from ASM cells, we then used neutralizing anti-IL-13 Ab to investigate whether IL-9-induced eotaxin1/CCL11 release on human ASM cells is mediated via IL-13-dependent pathways. Neutralizing polyclonal Abs anti-IL-13,
like the goat IgG control, had no effect on IL-9-induced eotaxin1/CCL11 release from ASM cells (Fig. 6A), but significantly reduced the release of IL-13-induced eotaxin1/CCL11 by ASM cells (Fig. 6). A similar result was obtained with anti-IL-4-neutralizing Ab (data not shown). Conversely, neutralizing anti-IL-9 mAb has no effect on IL-13-induced eotaxin1/CCL11 release from ASM cells (Fig. 6D). In contrast, IL-9-mediated eotaxin1/CCL11 release is significantly reduced by anti-IL-9 mAb (Fig. 6C). Taken together, these results suggest that IL-9 induction of eotaxin1/CCL11 is not mediated via IL-13 or IL-4.

IL-9 enhances eotaxin1/CCL11 expression at the promoter level

IL-9 has been previously shown to induce CC chemokines mRNA expression in epithelial cells (32). Then, we investigated whether IL-9 induction of eotaxin1/CCL11 in ASM cells is mediated through neosynthesis of mRNA expression. ASM cells were stimulated for 6 h with IL-9, IL-1β (both at 10 ng/ml), or medium alone, and analyzed by semiquantitative real-time RT-PCR. IL-9 and IL-1β induced 4.2- and 8.5-fold increase of eotaxin1/CCL11 mRNA, respectively, compared with unstimulated cells (Fig. 7A). In contrast to eotaxin1/CCL11 mRNA expression, TARC/CCL17

Table I. Relative expression of IL-9Rα in ASM cells area of human bronchial biopsiesa

<table>
<thead>
<tr>
<th>Asthmatic Patients</th>
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<th>Normal Control Subjects</th>
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<tr>
<td>Mean gray value ± SD</td>
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a Quantitative analysis of IL-9Rα expression (green fluorescence) by ASM cells area within bronchial biopsies was performed using ImageJ Software (written by W. Rasband, National Institutes of Health). The mean Gy value represents the mean intensity of the staining within ASM cells area.
mRNA could not be detected in IL-9, IL-1β, or medium-treated cells (Fig. 7B).

Then, we investigated the effect of inhibiting transcription on IL-9-induced eotaxin1/CCL11 mRNA and protein expression by ASM cells. Primary ASM cells were treated with Act D for 1 h before stimulation with IL-9 or IL-1β (10 ng/ml) for 24 h. Act D significantly abrogated eotaxin1/CCL11 protein and mRNA expression in IL-9-stimulated ASM cells (p < 0.01) (Fig. 8, A and B). The same magnitude of inhibition of eotaxin1/CCL11 mRNA and protein release was obtained in IL-1β-stimulated cells (Fig. 8, A and B). In contrast, Act D had no effect on eotaxin1/CCL11 release from unstimulated cells (Fig. 8A).

The results described above suggest that IL-9 induces eotaxin1/CCL11 mRNA expression. To test this possibility, ASM cells were then transiently transfected with luciferase-driven reporter construct containing 2.25-kb fragment of the eotaxin1 promoter, pGL-E02 (26). Compared with unstimulated cells, stimulation with IL-9 or IL-1β (both at 10 ng/ml) showed increased luciferase activity (1.5- and 2.2-fold, respectively) in primary human ASM cells transfected with pGL-E02 (Fig. 8C, n = 3). Similarly, IL-4 (10 ng/ml) induced a 3.2-fold increase in luciferase activity compared with unstimulated cells. Taken together, these data suggest that IL-9 induces eotaxin1/CCL11 at transcriptional level.

Discussion

The ASM has been typically described as contractile tissue, responding to proinflammatory mediators and neurotransmitters by contracting and responding to bronchodilators by relaxing (34). However, it has recently been recognized that the synthetic function of ASM cells may contribute to the perpetuation of airway wall inflammation (35, 36).

In this study, we first investigated whether human cultured ASM cells express the IL-9R. The expression of IL-9Rα was established by RT-PCR, flow cytometry, and immunofluorescence studies using human ASM cells cultured in vitro. This finding documents that IL-9R is synthesized by bronchial smooth muscle, but it cannot demonstrate whether IL-9R is expressed in vivo or whether it is functional. To address those questions, we evaluated IL-9R expression in bronchial biopsies of asthmatic and normal subjects. The expression of IL-9R was present in ASM cells from all asthmatics tested but not in normal subjects. This result presents the first evidence of IL-9R expression by ASM cells from asthmatics and suggests that IL-9-dependent activation of ASM cells occurs in vivo.

Then, we investigated the possibility that ASM cells via an IL-9-dependent pathway may contribute to inflammatory cells accumulation in allergic asthma. Our data demonstrate that IL-9 affects ASM cells eotaxin1/CCL11 release in a time- and dose-dependent manner, but has no effect on TARC/CCL17. Furthermore, in contrast to neutralizing Abs anti-IL-9, neutralizing anti-IL-13 or anti-IL-4 have no effect on IL-9-mediated eotaxin1/CCL11 protein release from ASM cells. These data suggest that IL-9-induced eotaxin release from ASM cells is not mediated via IL-4 or IL-13 pathway. Interestingly, IL-9 induction of eotaxin1/CCL11 mRNA and protein release in ASM cells was completely abrogated by pretreatment with Act D, which confirmed that IL-9 affects eotaxin1/CCL11 mRNA expression. Moreover, using transient transfection, we found that IL-9 enhances eotaxin1/CCL11 promoter activity. Taken together, this study identifies ASM cells as another target for IL-9 and that ASM cells participate in airway eosinophilia via an IL-9-dependent pathway.

Airway eosinophilia was observed in transgenic mice that overexpress IL-9 selectively within the lungs, and in naive mice administered with rIL-9 intratracheally (14, 15). Studies from systemically expressing transgenic mice showed that the overexpression of IL-9 enhanced an eosinophilic inflammation during challenge with Ag (14). These studies suggested that IL-9 could directly influence eosinophilia. To clarify the role of ASM cells in this process, we further determined whether IL-9 was able to influence the expression of the specific chemotactic factors for...
human eosinophil, particularly eotaxin1/CCL11. Eotaxin1/CCL11 is a C-C chemokine originally discovered as the predominant eosinophil chemoattractant in the airway of Ag-challenged guinea pigs (37). It mediates eosinophil migration via the CCR-3 and also has chemotactic activity for basophils and T cells, especially Th2 cells (38). In our study, we found that IL-9 alone induces strong release of eotaxin1/CCL11 from ASM cells. This effect is to a great extent similar to eotaxin1/CCL11 induction by IL-4 and IL-13 (Figs. 4 and 6), yet provides support for the involvement of IL-9 on inducing airway eosinophilia. Our data are in discrepancy with recent evidence showing that IL-9 alone has no effect on eotaxin1/CCL11 release from tracheal smooth muscle cells (39). Because in this study, we clearly show that bronchial smooth muscle from asthmatic subjects, but not from normal control subjects, express IL-9R it is possible that tracheal smooth muscle cells used by Baraldo et al. (39) lack constitutive IL-9R, at least on the cell surface. One can speculate that cytokines such as TNF-α or IL-13 may induce surface IL-9R in tracheal smooth muscle cells (39).

We found also that IL-9, at different concentrations and time points, failed to induce another C-C chemokine TARC/CCL17 from the same primary ASM cells as well as macrophage-derived chemokine MDC/CCL22 (data not shown). A similar scenario has recently been shown in bronchial epithelium of asthmatic subjects and epithelial cell lines that produce TARC/CCL17 but not macrophage-derived chemokine (40). TARC/CCL17 has been shown to elicit a selective migratory response in Th2 cells (41) and increased expression has been shown in murine experimental models of asthma (42, 43). Moreover, neutralizing of TARC/CCL17 resulted in attenuation of pulmonary allergic inflammation (42, 43). On the basis of our results, one can consider that ASM cells activation via the IL-9 pathway contributes preferentially to eosinophil recruitment.

Recently, IL-13 and IL-4 have been shown to induce selectively eotaxin1/CCL11 release from ASM cells (44). Hence, we investigated whether IL-9 may induce IL-4 or IL-13 release, which in turn through an autocrine pathway led to eotaxin1/CCL11 expression and release from ASM cells. In our study, however, we found that IL-9 induction of eotaxin1/CCL11 from ASM cells is

**FIGURE 6.** IL-9-mediated ASM cells eotaxin1/CCL11 protein release is significantly inhibited by neutralizing anti-IL-9 and is independent on IL-13. A and D. Growth-arrested human ASM cells were first preincubated with neutralizing goat anti-IL-13 or mouse anti-IL-9 (1 μg/ml) for 1 h before stimulation with IL-9 or IL-13 (both at 10 ng/ml) for 24 h. Goat IgG and mouse IgG1 mAb were used as isotype control. Eotaxin1/CCL11 accumulation in the medium was measured by ELISA as described in Materials and Methods. Data represent the mean ± SD of triplicate values from three independent experiments using CM from ASM cells. *, p < 0.01 as compared with IL-13-stimulated cells; ψ, p > 0.05 as compared with IL-13 or IL-9-stimulated cells.

**FIGURE 7.** Effect of IL-9 eotaxin1/CCL11 and TARC/CCL17 mRNA expression. A. ASM cells were stimulated with IL-9 or IL-1β (both at 10 ng/ml) for 6 h, then eotaxin1/CCL11 mRNA was analyzed by real-time RT-PCR. Results are expressed as fold increase over unstimulated cells. B. One sample from A analyzed by gel electrophoresis following real-time RT-PCR with TARC/CCL17 primers. Data represent geometric mean ± SD of three independent experiments from different donors. *, p < 0.01.
PCR, respectively, as described in Materials and Methods mRNA (53x358)/H11569 donors. (53x348)/H11006 significantly enhances eotaxin1/CCL11 promoter activation. IL-9 alone signiﬁcantly enhances eotaxin1/CCL11 promoter activity. Moreover, transfection data showed that IL-4/IL-13 and IL-9 effects on ASM cells function. Studies are needed to investigate the role and relationship between the lung, rather than inhibitory activity on T cells (47). More and IL-13) may be explained by reduced recruitment of T cells to lar lavage of OVA-sensitized and -challenged mice (47). This 9-neutralizing Ab reduced IL-4 and IL-13 levels in bronchoalveolar lavage. A recent study in a mouse model of asthma showed that anti-IL-9 does not affect IL-4, or IL-13 T cell production (45, 46). However, IL-9 induction of chemokines on epithelial cells does not rely on IL-9 on IL-4 and IL-13 production (32, 45, 46). Notably, in vitro not mediated through the production of IL-13 or IL-4, thus suggesting that IL-9 has a direct effect on eotaxin1/CCL11 expression. Conversely, blocking IL-9 has no effect on IL-13 or IL-4) induction of eotaxin1/CCL11 release in our system. Our data support recent in vivo and in vitro investigations that show no effect of IL-9 on IL-4 and IL-13 production (32, 45, 46). Notably, in vitro IL-9 induction of chemokines on epithelial cells does not rely on IL-4 or IL-13 production (32). Moreover, IL-9 deficiency in mice does not affect IL-4, or IL-13 T cell production (45, 46). However, a recent study in a mouse model of asthma showed that anti-IL-9-neutralizing Ab reduced IL-4 and IL-13 levels in bronchoalveolar lavage of OVA-sensitized and -challenged mice (47). This inhibitory effect of neutralizing anti-IL-9 on Th2 cytokines (IL-4 and IL-13) may be explained by reduced recruitment of T cells to the lung, rather than inhibitory activity on T cells (47). More studies are needed to investigate the role and relationship between IL-4/IL-13 and IL-9 effects on ASM cells function. Of interest is the finding that IL-9 affects eotaxin1/CCL11 expression at mRNA level. Moreover, transfection data showed that IL-9 alone signiﬁcantly enhances eotaxin1/CCL11 promoter activ- ity. Our results are in conﬂict with recent data showing that IL-9 alone has no effect on eotaxin1/CCL11 promoter activity (39). This can be explained ﬁrst by the difference of cellular source used in our study compared with Baraldo et al. (39). Secondly, it is possible that tracheal smooth muscle cells are deﬁcient of membrane-band IL-9R or express nonfunctional truncated forms (48) by tracheal smooth muscle cells. Thirdly, the IL-9–signaling pathway may differ between tracheal vs bronchial smooth muscle cells. The signal transduction in IL-9–mediated responses has been shown to be dependent on the JAK-STAT pathway. Previous studies have shown a constitutive association of JAK-1 with the IL-9R, and noted the presence of IL-9-induced activated complexes containing STAT-1, STAT-3, and STAT-5 (49, 50). Moreover, other studies have shown that IL-9 induced tyrosine phosphorylation of insulin receptor substrate-2 through JAK kinases activity (51). In tracheal ASM cells, the ERK/MAPK pathway has recently been shown to be involved in IL-9 activation (39). Preliminary data from our laboratory revealed that IL-9-induced eotaxin1/CCL11 is not dependent on STAT-5 activation. More studies are needed to characterize the IL-9–signaling pathway in ASM cells. In conclusion, our work provides the ﬁrst evidence in vitro and in vivo that ASM cells express IL-9R and that ASM cells via IL-9 participate in airway inﬂammation, particularly eosinophilia.

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References

FIGURE 8. IL-9 enhances eotaxin1/CCL11 expression at the transcriptional level. Cells were pretreated first with Act D for 1 h, then stimulated with IL-9, IL-1β (both at 10 ng/ml), or vehicle. Supernatants (A) and mRNA (B) were harvested at 24 h and analyzed by ELISA and real-time PCR, respectively, as described in Materials and Methods. Data represent geometric mean ± SD of three independent experiments from different donors. *, p < 0.01. C, ASM cells were transfected with eotaxin1/CCL11 promoter luciferase construct and incubated for 24 h. Transfected cells were then stimulated with 10 ng/ml IL-9, IL-1β, or IL-4. After 12 h, cell extracts were prepared and luminescence was measured. Data was normalized according to the Renilla luciferase activity. Geometric mean ± SD of three experiments.


