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Human Airway Smooth Muscle Cells Express the High Affinity Receptor for IgE (FcεRI): A Critical Role of FcεRI in Human Airway Smooth Muscle Cell Function

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Several reports suggest that activated airway smooth muscle (ASM) cells are capable of generating various proinflammatory mediators, including cytokines and chemokines. However, little is known about the mechanism involved in this process. In this regard, we have examined the expression and the role of the high affinity IgE receptor (FcεRI) by ASM cells. Human ASM cells were found to constitutively express transcripts coding for α, β, and γ subunits of FcεRI. Flow cytometry and Western blot analysis confirmed the expression of FcεRI α-chain protein. Interestingly, FcεRI α-chain immunoreactivity was also demonstrated in smooth muscle within bronchial biopsies of asthmatic subjects. Cross-linking of FcεRI induced mobilization of free calcium in ASM cells, one of the critical signals to trigger smooth muscle contraction. Furthermore, cultured ASM cells released IL-4, IL-13, IL-5, and eotaxin but not IFN-γ, when sensitized with IgE followed by anti-IgE Ab cross-linking. The addition of anti-FcεRI α-chain Abs directed against IgE binding site inhibited this release. Taken together, these results suggest a potential new and important mechanism by which ASM cells may participate in airway inflammation and bronchoconstriction associated with allergic asthma. *The Journal of Immunology,* **2005, 175: 2613–2621.**

Asthma is a chronic inflammatory disease of the bronchial airways that has been increasing in prevalence, morbidity, and mortality over the last four decades. Considerable evidence demonstrates that airway inflammation is a major factor in the pathogenesis of asthma, often associated with bronchial hyperresponsiveness and correlated with disease severity (1). The inflammatory component of this disease includes an increased number of activated T lymphocytes, mast cells, neutrophils, and eosinophils within the airway lumen and bronchial submucosa (2). Release of mediators from infiltrating inflammatory cells in the airway mucosa has been proposed to contribute directly or indirectly to the changes observed in airway structure and function (1). This includes induction of myocyte contraction and smooth muscle hypertrophy (increase in cell size) and/or hyperplasia (increase in cell number) leading to increased bronchial muscle mass (1, 3). These structural changes are hallmark features of airway remodeling, a phenomenon believed to have profound consequences for airway function (4)

Classically, airway smooth muscle (ASM)[3] cells have been regarded as contractile tissue, responding to proinflammatory mediators, neurotransmitters, and bronchodilators to regulate airway caliber directly (5). However, recently it has been recognized that ASM cells are also a rich source of proinflammatory cytokines, chemokines, and growth factors (4, 6). Indeed, cultured ASM cells have been shown to express a number of cytokines (Th1-type: IL-2, IFN-γ, IL-12; and Th2-type: IL-5, IL-6, GM-CSF) and high level of RANTES, eotaxin, IL-8, and IL-11 that may perpetuate airway inflammation and the development of airway remodeling in vivo (6).

Cumulative data has clearly demonstrated that serum IgE plays an important role in the pathogenesis of smooth muscle hyperreactivity. Bronchial hyperreponsiveness was shown to be associated with serum IgE levels and to be transferable from asthmatic to non-asthmatic subjects by IgE-rich serum (7). Incubation of smooth muscle cells with serum from atopic individuals that contains high levels of IgE was shown to induce hyperreactivity in isolated airway preparations (8) and IgE cause abnormal smooth muscle contractile function through binding to the smooth muscle cell membrane and subsequent hyperpolarization (9). It has been reported that sensitized ASM cells express the low affinity IgE receptor for IgE (FcεRII/CD23) (11). Furthermore, a recent study demonstrated that IgE sensitization of ASM cells elicits the sequential autocrine release of IL-13 and IL-5 by the sensitized ASM cells itself (10). Importantly, this Th2-type autocrine response may contribute to the changes in ASM cell responsiveness that characterize the atopic asthmatic phenotype, including heightened agonist-mediated constrictor responsiveness and impaired β-adrenoceptor-mediated ASM cell relaxation (11). Although this reveals an important ability of these cells to respond to IgE stimuli, to date

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[3] Abbreviations used in this paper: ASM, airway smooth muscle; B/TSM, bronchial/tracheal smooth muscle; MFI, mean fluorescence intensity.

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expression of the high affinity IgE receptors (FcεRI) by ASM cells has not been investigated.

In the present study, we report that FcεRI is expressed in cultured human ASM cells. A strong signal for FcεRI immunoreactivity was also observed in vivo in smooth muscle cells in asthmatic airways. In addition, functional studies demonstrate that cross-linking of IgE bound to its receptors induces both a rapid, transient mobilization of intracellular calcium, and the release of inflammatory mediators including Th2 cytokines and chemokines. These observations suggest a new mechanism by which IgE and ASM cells participate in airway inflammation and hyperresponsiveness.

Materials and Methods

Monomeric recombinant human IgE was obtained from Diatek. Biotin conjugated horse anti-mouse IgG Abs were obtained from Vector Laboratories. Goat anti-mouse IgG1 was purchased from Southern Biotechnology Associates. Murine anti-human FcεRIα-chain mAb, mAb15–1 (mouse IgG1), which recognizes the IgE-binding site (12), was kindly donated by Dr. J. P. Kinet (Harvard Medical School, Boston, MA) and purchased from Upstate Biotechnology. Rabbit polyclonal Ab directed against the extracellular region of human FcεRI α-chain was purchased from Upstate Biotechnology. FITC-labeled swine anti-rabbit $F(ab')_2$ was purchased from DakoCytomation. Rat anti-mouse IgG FITC labeled was obtained from Jackson ImmunoResearch Laboratories. Fura 2-AM was purchased from Molecular Probes. FBS was obtained from HyClone. Unless stated otherwise, all other reagents were obtained from Sigma-Aldrich.

Study population

Asthmatic patients were recruited from the asthma clinic (Chest Hospital, Montreal) and their characteristics were previously described (13). Six patients with positive skin tests to at least one aeroallergen and diagnosis of asthma, as defined by the American Thoracic Society, were studied (14). 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 5 pack/year history of smoking. Patients 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 (13, 15).

Cell culture

Human ASM cells from two sources were used. Bronchial/tracheal smooth muscle (B/TSM) cells were purchased from Cambrex. These cells stained positively for α-smooth muscle actin and negatively for factor VIII, CD45, and CD3. B/TSM cells were grown as recommended by the supplier in their optimal medium (SmGM-2; Cambrex) containing 5% FBS, insulin (5 ng/ml), epidermal growth factor (10 ng/ml; human recombinant), fibroblast growth factor (2 ng/ml; human recombinant), gentamicin (50 ng/ml), and amphotericin B (50 ng/ml) at 37°C, 5% CO_2.

The second source of cells was human ASM cells (P2 to P5), which were obtained from central airway segments (0.5–1.0 cm diameter) in pathologically uninvolved segments of resected lung specimens using isolation methods described previously (16). Human ASM cells were grown at 37°C in DMEM supplemented with 10% FBS, sodium pyruvate (1 mM), L-glutamine (2 mM), non-essential amino acid mixture (1:100), gentamicin (50 μg/ml), and amphotericin B (1.5 μg/ml). Fresh medium was replaced every 2 days. These cells retain smooth muscle-specific actin expression and have the requisite receptor/second-messenger systems necessary to support both contractile and relaxant responses (13). B/TSM and ASM cells grow with the hill-and-valley appearance characteristic of smooth muscle in culture and are elongated and spindle shaped with a central nucleus.

RT-PCR analysis

Total B/TSM cells RNA was extracted from serum-fed confluent cultures using Trizol reagent (Invitrogen Life Technologies). RT was performed by using 2 μg of total RNA of B/TSM cells in a first strand cDNA synthesis reaction with Moloney murine leukemia virus reverse transcriptase as recommended by the supplier (Invitrogen Life Technologies). Oligonucleotides specific for α, β, γ, and CD23 sequences on either side of a splice junction were used in the PCR to preclude amplification of possible contaminating genomic DNA. Oligonucleotide primers were designed on the basis of the published sequences (17). The FcεRI α-chain oligonucleotides were: 5′ primer: 5′-TACAGTAATGTTGAGGGGCTCAG-3′; 3′ primer: 5′-CTGTTCTTCGCTCCAGATGGCGTT-3′; internal primer: 5′-CCTG TACACATCCCAGTTCCTCCAACCAT-3′. The FcεRIβ-chain primers were: 5′ primer: 5′-GGACACAGAAAAGTAATAGGAGAG-3′; 3′ primer: 5′-GATCAGGATGGTAATTCCCGTT-3′; internal primer: 5′-TTT TCATCATTAAGCAGGTTATCCATT-3′. The FcεRII primers were: 5′ primer: 5′-CTGTGGCACTGGGACACCACA-3′; 3′ primer: 5′-TGTGT GCAACACGTGCCCTGAA-3′; CD23 internal primer: 5′-TGGACTGG GATTTCTGCGCCAT-3′. PCR was conducted as described previously (17). Mast cell tryptase β1 oligonucleotides were: 5′ primer: 5′-TCAG CAGGATCATCG TGCAC-3′; 3′ primer: 5′-TGGGGACATAGTGGTG GATC-3′; GAPDH oligonucleotides were: 5′ primer: CCGGAGGGGC CATCCACAGTCT-3′; 3′ primer: AGCAATGC TCCTGCACCACC AAC-5′.

Southern blot analysis

Bands were transferred to nylon membranes and Southern analysis was performed with an internal primer for FcεRIα and β as well as CD23/FcεRII (12, 13) to verify the specificity of the PCR product. Oligonucleotide probes were labeled with 3-deoxy digoxigenin-labeled ATP using terminal transferase enzyme. The blots were prehybridized for 2 h at 42°C in hybridization solution (50% formamide, 5% SSC, 0.1% sodium lauroylsarcosine, 0.1 mg/ml poly(A), 0.02% SDS and 2% blocking reagent) (Boehringer Mannheim). Hybridization was performed with digoxigenin-labeled oligonucleotide probe for 18 h at 42°C. The blots were washed at high stringency conditions: three times at room temperature in 2× SSC, 0.05% SDS, and twice at 60°C in 0.1× SSC, 0.05% SDS for 30 min each. The blots were equilibrated in washing buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.3% v/v Tween 20) for 5 min, then incubated with goat anti-digoxigenin-alkaline phosphatase Fab (1:20,000) in blocking solution (Boehringer Mannheim) for 30 min, washed twice with washing buffer at room temperature for 15 min each. After equilibration with detection buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5) for 2 min, the blots were incubated for 5 min with CPD-Star diluted in detection buffer (1:100). For detection of chemiluminescent signal, the blots were exposed to x-ray film for 20 min at room temperature.

Immunostaining and immunofluorescence studies

Immunohistochemistry was performed as previously described (13). Fresh frozen sections (5 μm) and cytospin slides were saturated with universal blocking solution for 10 min (DakoCytomation). Slides were incubated with anti-FcεRI mAb15–1 or isotype matched control (5 μg/ml) overnight at 4°C, washed twice with TBS followed by incubation for 1 h at room temperature with 5 μg/ml horse anti-mouse IgG biotin-conjugated (Vector Laboratories). Slides were then extensively washed with TBS and incubated with streptavidin-alkaline phosphatase for 30 min at room temperature. After washing with TBS, the slides were developed using Fast Red and counterstained with Mayer's hematoxylin. Positive cells stained red after development with Fast Red (Sigma-Aldrich). Omission of the primary Ab as well as staining with an isotype-matched control mAb was used for negative controls. For cytospin preparations, the slides were incubated with anti-FcεRI mAb15–1 followed by rat anti-mouse IgG FITC-labeled Abs and nuclei of cells were stained for 2 min with Hoechst 33258 (1 pg/ml) (Sigma-Aldrich). The slides were then covered with a Vectorshield antifading mounting medium obtained from Vector Laboratories and were visualized by using a confocal fluorescence microscope (Bio-Rad).

For cytokine expression, immunostaining was used as described above using cytospin slides of ASM cells from asthmatics and anti-IL-4, IL-5, IL-13 mAbs (R&D Systems) followed by horse anti-mouse IgG biotin conjugated and streptavidin-HRP. After washing with TBS, the slides were developed with DAB substrate kit and counterstained with Mayer's hematoxylin (Vector Laboratories).

Flow cytometry analysis

A suspension of primary cultured human ASM cells of asthmatics (10^5 cells in 100 μl of PBS/5% FCS) was prepared from serum-fed confluent cultures and then was incubated with gentle agitation for 1 h on ice with mAb anti-FcεRIα Abs (17) or mAb control (MOPC21) at final concentration of 10 μg/ml. The cells were washed twice with PBS/2% FCS and incubated in the dark for 30 min on ice with rat FITC-conjugated anti-mouse IgG Abs (1:200). Similarly, analysis of surface expression FcεRI by human ASM cells was also performed with polyclonal Ab anti-FcεRI α-chain, or rabbit IgG used as negative control, both at 5 μg/ml followed by swine anti-rabbit $F(ab')_2$ (1:100) under the same condition described above. The cells were washed three times with PBS/2% FCS, resuspended

in 500 μl of PBS, and analyzed on FACScan. FACS analysis was performed with Cellquest software (BD Biosciences). The results are presented as percentage of positive cells and mean fluorescence intensity (MFI) as described previously (17).

Western blot analysis

Adherent B/TSM cells and ASM cells were washed in prechilled PBS and solubilized in lysis buffer (50 mM HEPES, pH 7.5, 150 mM, 5 mM NaF, 10 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, and protease inhibitors (Boehringer Mannheim) for 30 min at 4°C. Supernatants were collected by centrifugation and assessed qualitatively by immunoprecipitation followed by immunoblot as described previously (17). Extracted proteins were incubated with 2 μg of human IgE recombinant proteins for 3 h at 4°C in lysis buffer. IgE receptor complexes were incubated for 1 h with 5 μg of the goat anti-human IgE Abs adsorbed to protein A/G-Sepharose beads (Amersham Biosciences). The beads were washed with lysis buffer, and the immunoprecipitates resuspended in 2× concentrated Laemmli sample buffer containing (1 M Tris, 25% glycerol, 0.5% SDS, 15% 2-ME, 0.1 mg/ml bromphenol blue) and boiled for 10 min at 70°C. Finally, the samples were separated by standard SDS-PAGE under reducing conditions and blotted to polyvinylidene membranes (Millipore). The blots were blocked in TBS containing 1% nonfat milk, 1% BSA (Sigma-Aldrich), 0.05% Tween 20, and anti-FcεRI α-chain (mAb15–1) was added in the same buffer at 4°C for 12 h. Biotin-conjugated horse anti-mouse Ig (1:1000) was added for another hour and washed three times in TBS containing 0.05% Tween 20 followed by incubation for 45 min with streptavidin-alkaline phosphatase at 1:1000 (Boehringer Mannheim). The blots were revealed by ECL as recommended by the supplier. To show that the detection Ab does not cross-react with any of the Abs present on the gel, other than the mAb15–1, mouse irrelevant mAb was used as a negative control in the same conditions.

Measurement of intracellular free Ca^{2+}

For calcium measurements, coverslips with confluent serum-fed ASM cells primary cultures were rinsed twice with prewarmed (37°C) HEPES-buffered (10 mM, pH 7.4) HBSS/1% BSA, then incubated for 1 h at 37°C in the same buffer containing the calcium-sensitive dye, fura 2-AM (10 μM) as previously described (18). Thereafter, the fura 2-AM-loaded cultures were washed twice with HEPES-HBSS and further incubated in the dark at room temperature for 30 min before measurement of intracellular calcium. Glass slides were then mounted on an inverted microscope (Olympus IX70) equipped with an OlymPix TE3/A/S digital camera controlled through a PC workstation. Cells were alternatively exited at 340 and 380 nm using a filter wheel controlled with a Lambda 10 filter wheel (Sutter Instruments). Emitted fluorescence (510 nm) was acquired for 350 ms at each excitation wavelength and used to calculate calcium concentrations (in nanomolar concentration) at each pixel from an in vitro calibration curve of known free Ca^{2+} (0–1.35 μM) and pentapotassium fura 2 (50 μM). An Olympus UAPO/340 20X/0.75 objective was used for all studies and image size was set to 540 × 540 pixels. Calcium responses within individual cells were determined using UltraView version 4.0 software (Olympus LSR) by circumscribing single myocytes and spatially averaging fura 2 fluorescence in each cell. At the beginning of each experiment each chamber contained 200 μl of HEPES-HBSS, the cells were then primed by adding an equal volume of HEPES-HBSS containing 10 μg/ml of either recombinant human IgE or anti-FcεRI 15–1 (both at 10 μg/ml) for 30 min. After rinsing, intracellular free Ca^{2+} was recorded for 30 s to establish a baseline. Then cross-linking Ab, goat anti-human IgE or goat anti-mouse IgG1 was added and intracellular free Ca^{2+} was recorded for at least 200 s to characterize peak and plateau responses. As a negative control, cells were incubated with an irrelevant IgG1 mAb (MOPC21), then cross-linked with goat anti-mouse IgG1. Acetylcholine was used as positive control. Studies were performed in duplicate using three cell lines each acquired from a different donor.

Cell stimulation and ELISA

Primary cultured human ASM cells and B/TSM cells in passages 2–5 were growth to confluence in the appropriate medium. Thereafter, using the same donor cell preparations, the cells were growth arrested by FBS deprivation for 48 h and stimulated in fresh serum-free medium containing IgE (1, 50, 500, 5000, and 25,000 ng/ml) for 4 days at 37°C. Cells were extensively washed and then stimulated with IgE (5 μg/ml) for 30 min at 37°C followed by goat anti-human IgE Ab (1 μg/ml) for 12 h. In inhibition studies, cells were preincubated at 4°C for 1 h with Abs directed against FcεRI α-chain or control Abs before stimulation with IgE and goat anti-human IgE antibodies. Supernatants were collected from culture flasks, centrifuged at 1200 rpm for 7 min at 4°C to remove cellular debris, and stored at −80°C until use. The cytokine and chemokine released in the supernatants were measured using Duoset development ELISA kits for IL-4, IL-5, IL-13, IFN-γ, and eotaxin (R&D Systems). The sensitivity limits of these kits are 3 pg/ml for IL-4, 3 pg/ml for IL-5, 6 pg/ml for IL-13, 8 pg/ml for IFN-γ, and 5 pg/ml for eotaxin. To investigate the effect of blocking FcεRI in mediator release, anti-FcεRI mAb15–1, or control mAb (10 μg/ml) were added for 1 h to the cells before the sensitization with IgE and challenged with goat anti-human IgE.

Statistical analysis

Statistical significance was determined using a Mann-Whitney *U* test and paired Student *t* test. All statistical tests were performed using Instat Software 2.0 (GraphPad Software). Values of $p < 0.05$ were considered statistically significant.

Results

Expression of FcεRI receptor mRNA by human ASM cells

Previous studies have demonstrated that FcεRI is expressed either as tetrameric ($\alpha\beta\gamma_2$) multisubunit complex in mast cells and basophils or as a trimeric ($\alpha\gamma_2$) complex on monocytes, Langerhans, and dendritic cells (19). The expression of FcεRI α, β, γ mRNA in the cultured B/TSM cells was examined using RT-PCR and Southern blot analysis (Fig. 1*A*). RNA preparation from cultured ASM cells revealed mRNA expression of FcεRIα subunit (Fig. 1*Aa*) β subunit (Fig. 1*Ac*), and FcεRI γ subunit (Fig. 1*Ae*). Expression of FcεRII/CD23 mRNA by ASM cells was also detected (Fig. 1*Ag*), as previously reported (10). Furthermore, a similar pattern was detected in PBMC used as positive control (Fig. 1*Aa–Ah*)

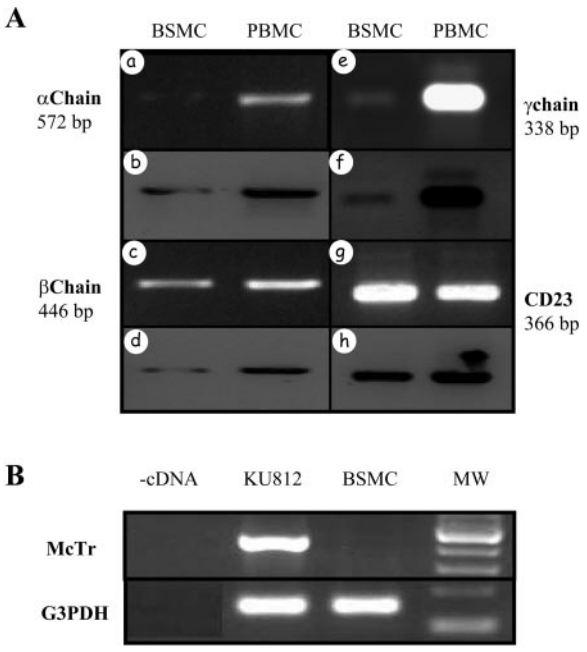

FIGURE 1. FcεRI α, β, and γ-chain mRNA expression in ASM cells. RT-PCR was performed using total RNA purified from confluent B/TSMC (P2-P5) cultured in serum-free medium. *A*, RNA obtained from human PBMC of asthmatic patients was used as positive control. Putative FcεRI α (*a*), β (*c*) γ (*e*) subunits and CD23 (*g*) bands (572, 446, 338, and 366 bp, respectively) were transferred to nylon membranes and hybridized with a digoxigenin-labeled internal oligonucleotide specific for FcεRI α (*b*), β (*d*), γ (*f*) subunits, and CD23 (*h*) bands as described in *Materials and Methods*. *B*, Total RNA was extracted from B/TSM cells and amplified by RT-PCR for tryptase β1 mRNA as described in *Materials and Methods*. As a positive control, RNA from KU812 cell line was used. As a negative control, RNA was omitted from the RT and PCR amplification (−cDNA). Results are representatives of three independent experiments performed under the same conditions.

and in ASM cells obtained from bronchial biopsies of two patients (data not shown). As shown in Fig. 1, the specificity of the RT-PCR signal was confirmed by Southern blotting using internal oligonucleotides specific for FcεRIα (Fig. 1*Ab*), β subunit (Fig. 1*Ad*), γ subunit (Fig. 1*Af*), and CD23 (Fig. 1*Ah*). To exclude the possibility that a nonspecific expression of FcεRI transcripts by a small number of mast cells, present in our preparations, could have contributed to the increase in FcεRI RNA expression, tryptase RNA expression was examined using RT-PCR. These studies demonstrated that tryptase mRNA was absent in our smooth muscle RNA preparations (Fig. 1*B*). Therefore, the expression of FcεRI α-chain is selective and not just a coincidence of increased mast cell numbers.

FcεRI is expressed in vivo in asthmatic bronchial biopsies

To gain more insight into the expression of the high affinity IgE receptor by human ASM cells in vivo, immunohistochemistry in bronchial biopsies from asthmatic subjects was performed using anti-FcεRI α-chain mAb. As shown in Fig. 2, *a* and *b*, FcεRI α-chain immunoreactivity was detected in smooth muscle cells from all asthmatic subjects who were investigated ($n = 6$). No immunoreactivity was found in any cell type when the first Ab was omitted or replaced by an isotype control mAb (Fig. 2*c*).

Because eosinophils and mast cells have been described in the vicinity of ASM cells, we further used immunofluorescence technique on isolated ASM to demonstrate FcεRI α-chain within the cells. In Fig. 2*d*, ASM from asthmatic subjects show a positive signal with anti-FcεRI mAb (mAb15–1). No cells were labeled when incubated with isotype control mAb (Fig. 2*e*).

FcεRI α-chain protein expression by ASM cells

In light of the above finding, we further investigated the surface expression of FcεRI by human ASM cells. Using flow cytometry with a mAb directed against human FcεRI α-chain (mAb15–1), analysis of FcεRI surface expression revealed positive signal in subpopulation of human ASM cells (Fig. 3*A*). This expression pattern was variable according to individual donors ranging from 31% to 38% with a mean of 34% (MFI = 14, $n = 3$) and was independent of passage number (P3 to P5 analyzed). Interestingly, similar levels of FcεRI surface expression in human ASM cells were detected with rabbit anti-FcεRIα-chain polyclonal Abs (Fig. 3*B*).

The protein expression of FcεRI α-chain by ASM and B/TSM cells was then investigated using immunoprecipitation followed by Western blot analysis. A band at the expected molecular mass (~46 kDa) corresponding to the FcεRIα-chain was detected on B/TSM cells and PBMC used as positive control (Fig. 4). No band could be detected in ASM cells and PBMC when the Western blot was performed with mAb15–1 without prior IgE immunoprecipitation (Fig. 4*B*) or when the mAb15–1 was replaced by a control mAb (Fig. 4*A*). Taken together, these results demonstrated that human smooth muscle cells express FcεRIα of both the protein and mRNA levels.

Cross-linking of FcεRI induces intracellular free Ca^{2+} fluxes in ASM cells

Because smooth muscle contraction depends on an increase in intracellular calcium concentration $[Ca^{2+}]_i$ and the subsequent activation of myosin L chain kinase (20), we investigated whether FcεRI activation induces intracellular calcium flux in human ASM cells. Cross-linking of FcεRI receptors treatment of ASM cells with human IgE or anti-FcεRI α-chain mAb (15–1) followed by goat anti-mouse IgE or anti-mouse IgG1 induced marked mobilization of intracellular free Ca^{2+} within 5 to 10 s; a peak of cytosolic concentrations of Ca^{2+} (mean peak $[Ca^{2+}]_i = 170$ nM) was attained between 10 and 30 s, and returned to baseline values thereafter (Fig. 5, *A* and *B*). There was no increase of Ca^{2+} when irrelevant mAb instead of anti-FcεRI mAb were used, but responded well to acetylcholine (mean peak $[Ca^{2+}]_i = 370$ nM) (Fig. 5, *C* and *D*). These data show unequivocal evidence for the expression of functional FcεRI by cultured human ASM cells.

FcεRI is involved in IgE-mediated release of cytokines and chemokines from ASM cells

Increased expression of cytokines and chemokines within the airway is a common feature of allergic inflammation (21). To verify that the FcεRI expressed by human ASM and B/TASM cells was involved in this process, we examined whether IgE-dependent activation of human ASM and B/TASM cells could induce the release of eotaxin and Th2 cytokines (IL-4, IL-5, and IL-13). To induce proasthmatic-like changes in smooth muscle cells, we therefore assessed whether preincubation of these cells with IgE might up-regulate FcεRI expression. Indeed, incubation of

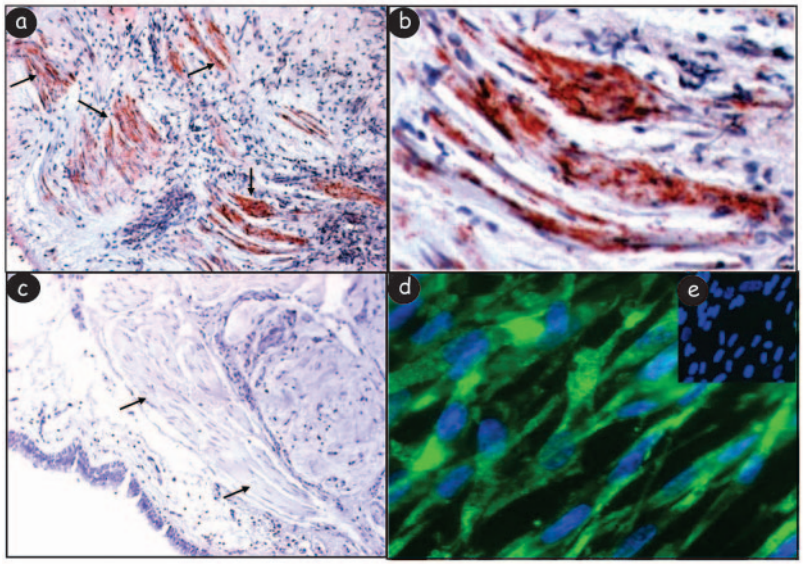

FIGURE 2. Detection of FcεRI α-chain immunoreactivity on bronchial smooth muscle tissue from asthmatic subjects. *a*, transverse section of a large airway in a subject with asthma, demonstrating FcεRI-positive immunoreactivity using anti-FcεRI α-chain mAb15–1 (large arrows). *b*, High-power magnification of the same transverse section of a large airway in a subject with asthma, demonstrating FcεRI immunoreactivity in bundles of smooth muscle by the streptavidin-alkaline phosphatase method of immunostaining. Cryostat sections were cut from frozen lung tissue from asthmatic subjects. Slides were incubated with anti-FcεRI α-chain mAbs, the appropriate secondary antibodies, and a tertiary layer of streptavidin-alkaline phosphatase. Sections were developed with Fast Red substrate and positive cells stained red than surrounding cells. *c*, No staining was detected in sections labeled with the negative control mAb IgG1. *d*, Immunofluorescent staining of FcεRIα in unstimulated ASM cells of asthmatic patient. *e*, as control, no signal was seen with an isotype-matched control mAb on ASM cells. Similar results were obtained in three other experiments.

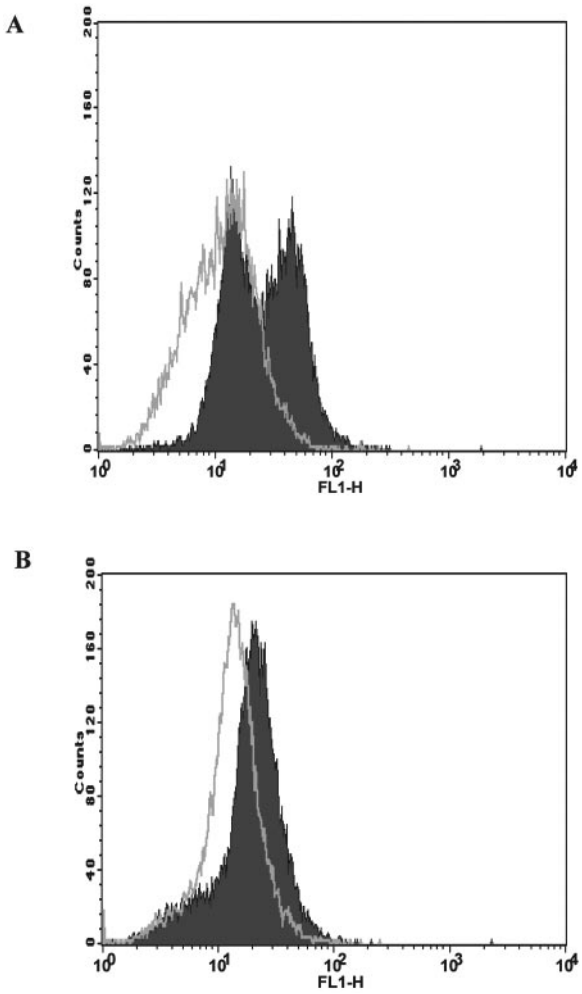

FIGURE 3. Surface expression of FcεRI on human ASM cells. Confluent human ASM cells (P2-P5) obtained from two subjects cultured in serum-free medium were analyzed for the presence of cell surface FcεRI by flow cytometry using a mouse anti-human FcεRI α-chain Ab (mAb15–1) and rabbit polyclonal anti FcεRI α-chain (filled lines in *A* and *B*, respectively). As a negative control, cells were labeled with isotype-matched IgG1 (MOPC-21) or rabbit IgG. Representative experiment out of three is shown. The percentage of positive cells or MFI was calculated by subtracting the isotype control from the specific signal.

B/TASM cells with IgE during 4 days increased the FcεRI α-chain mRNA expression (data not shown) and cytokine secretion by human smooth muscle cells upon FcεRI-dependent stimulation (Fig. 6). B/TASM cells were first incubated with a graded amount of IgE for 4 days, and then passively sensitized for 30 min with IgE, to allow saturation of FcεRI, followed by cross-linking with goat anti-human IgE. The kinetic study of cytokine secretion indicated that maximal secretion was obtained after 12 stimulations of B/TASM cells with IgE + anti-IgE Abs (data not shown). The release of eotaxin, IL-4, IL-5, and IL-13 in culture medium was evaluated after 12 h of culture. As shown in Fig. 6, IgE cross-linking induced the synthesis and release of IL-4, IL-5, IL-13, and eotaxin in a dose-dependent manner. Similar results were obtained with cytokine release being observed also in the supernatant of cultured human ASM cells without passive sensitization (IL-4, 85 ± 10; IL-5, 97 ± 5; IL-13, 60 ± 5; eotaxin, 650 ± 10) or after passive sensitization with IgE for 4 days (IL-4, 147 ± 8; IL-5, 173 ± 5; IL-13, 111 ± 5; eotaxin, 986 ± 12). However, IFN-γ release was not observed in the supernatants of the same preparations of B/TASM or ASM cells (data not shown). Treatment with IgE for 4 days before passive sensitization and challenge was essential for IgE-mediated B/TASM cells release of Th2 cytokines (IL-4, IL-5, and IL-13) and eotaxin, because Th2 cytokine and eotaxin release was mostly lacking when B/TASM cells were cultured without IgE (Fig. 6). The maximal effective dose of IgE was 5 μg/ml for eotaxin, whereas 25 μg/ml is more effective for Th2 cytokines release (Fig. 6). The dose of 5 μg/ml was then used in inhibition study. To investigate the protein expression of cytokines on ASM cells obtained from asthmatic patient, immunochemistry was performed with anti-IL-4, IL-5, IL-13, and eotaxin antibodies. Fig. 6*E* shows a specific staining for IL-4 in ASM cells of asthmatic subject. Substitution of the primary Ab with irrelevant mouse IgG eliminated the immunostaining confirming the specificity of the reaction (Fig. 6*F*). Similar data were obtained for IL-5, IL-13, and eotaxin (data not shown) (13).

In the light of the above finding, we then used anti-FcεRI α-chain blocking mAb to investigate the contribution of FcεRI receptor in mediating cytokine and chemokine release from B/TSM cells. B/TSM cells were pretreated with anti-FcεRI α-chain mAb15–1 (directed against IgE-binding site) (19) or mouse IgG1 isotype control at 10 μg/ml for 1 h and then incubated with IgE followed by anti-IgE. Pretreatment with anti-FcεRI α-chain mAb, but not with mouse IgG1 isotype control, significantly blocked IgE-mediated Th2 cytokines and eotaxin release from B/TASM cells (Fig. 7).

Discussion

In this study, we show that ASM cells express FcεRI. We also demonstrated in vivo expression of FcεRI in smooth muscle bundle within bronchial biopsies of asthmatic subjects. IgE-dependent activation of human ASM cells via the high affinity receptors for IgE (FcεRI), leads to Th2 cytokine and chemokine release (eotaxin, IL-4, IL-13, and IL-5) but not Th1 cytokines (IFN-γ). Furthermore, neutralizing anti-FcεRI α-chain mAb abrogates this

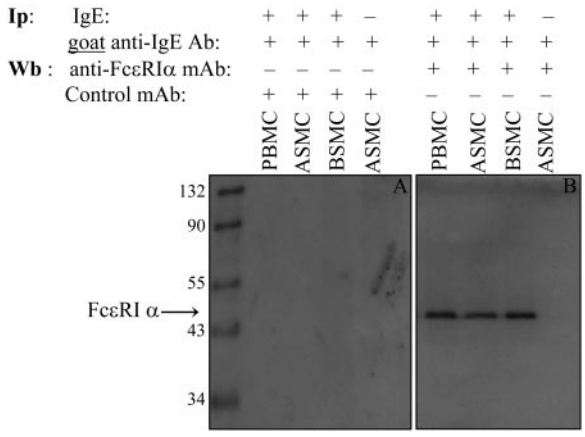

FIGURE 4. FcεRI α-chain protein expression in human B/TSM cells. Cell extract proteins of human PBMC and ASM cells of asthmatic patients or from human B/TSM cells (P2-P5) cultured in serum-free medium were first immunoprecipitated with IgE Fc fragment (*lanes 1–3*), followed by protein G-Sepharose-coated beads coupled goat anti-human IgE. The eluates were separated by SDS-PAGE (13%) and transferred to membrane. The membrane was incubated with control mAb (*A*) or mAb15–1 (*B*) followed by biotin-conjugated horse anti-mouse Ig and streptavidin-alkaline phosphatase. As a control for specificity, lysates that had not first been immunoprecipitated with IgE Fc fragment were also analyzed and no signal for FcεRI α-chain was detected. No band could be detected in ASM cells and PBMC extract proteins, when the mAb15–1 was replaced by a control mAb (*A*).

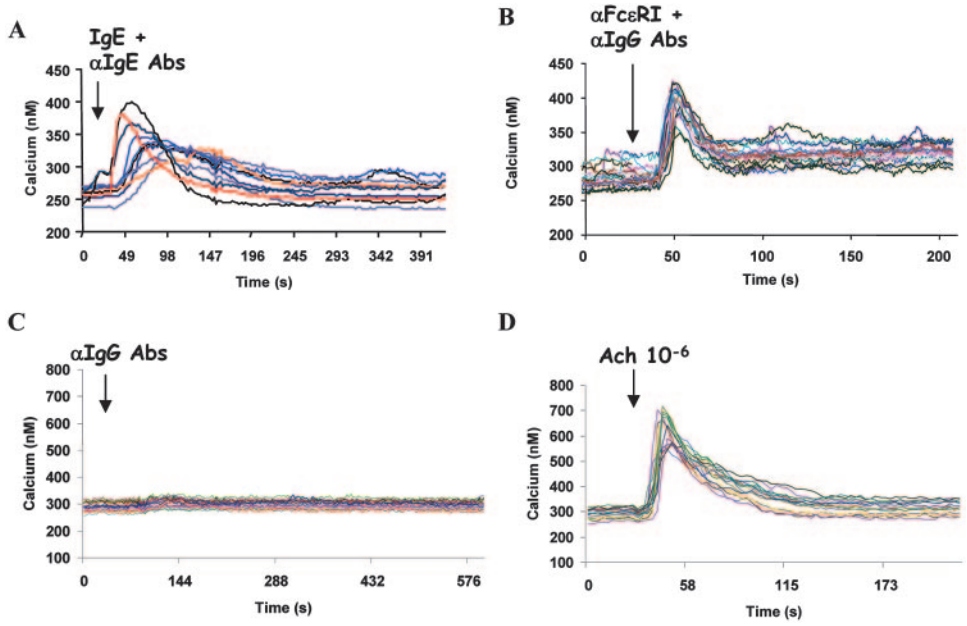

FIGURE 5. Activation of B/TSM cells via FcεRI induces intracellular calcium flux. For calcium measurements, confluent serum-fed human ASM cells were loaded with fura 2 as described in *Materials and Methods*. Cells were first sensitized with recombinant human IgE (*A*) or anti-FcεRI 15–1 (*B*) both at 10 μg/ml for 30 min. After a washing step, cross-linking was performed either with goat anti-human IgE (*A*) or goat anti-mouse IgG1 (*B*), and intracellular Ca^{2+} was measured for up to 300 s thereafter. Mouse IgG1 (MOPC21) (*C*) and acetylcholine (10^{-6} M) (*D*) were used as negative and positive controls, respectively. Data shown are representative of three experiments, and tracings from individual cells are included in each panel.

effect. Moreover, in vitro cross-linking of FcεRI induced a marked transient increase in intracellular Ca^{2+} that was qualitatively and quantitatively similar to the response induced by the contractile agonist acetylcholine. This observation clearly implicates a potential role for IgE-mediated ASM contraction. Collectively, our data provide new pathways to explain previous clinical studies that have found a close association between the prevalence of asthma (22), the degree of bronchial hyperresponsiveness, and serum-specific IgE levels (7, 23).

FcεRI was first described as a tetrameric complex ($\alpha\beta\gamma 2$) (19) and the structure of FcεRI varies according to the species. In mice, FcεRI β-chain is required for cell surface expression of FcεRI so that FcεRI is only expressed as $\alpha\beta\gamma_2$ tetramers on mast cells and basophils, whereas in humans FcεRI can be expressed as $\alpha\beta\gamma 2$ or $\alpha\gamma 2$ complexes (19). Using RT-PCR and Southern blot analysis, we found that cultured human B/TASM cells constitutively express the FcεRI α, β, and γ subunits. The expression of FcεRI α-chain was also confirmed at the protein level by using Western blot and flow cytometry analysis. Because we have not colocalized the three subunits at the cellular level, surface expression of FcεRI in the absence of β-chain as reported for other cells (19) cannot be excluded. Previous studies have demonstrated that monomeric IgE up-regulates FcεRI expression by a mast cell line (RBL-2H3) and human mast cells and basophils (19). Mast cells from IgE-deficient mice express low levels of FcεRI, which could be up-regulated by in vitro incubation of the cells with IgE or injection of IgE in vivo (24–26). Because elevated specific IgE is consistently present in airway secretions from human patients with bronchial asthma (27,

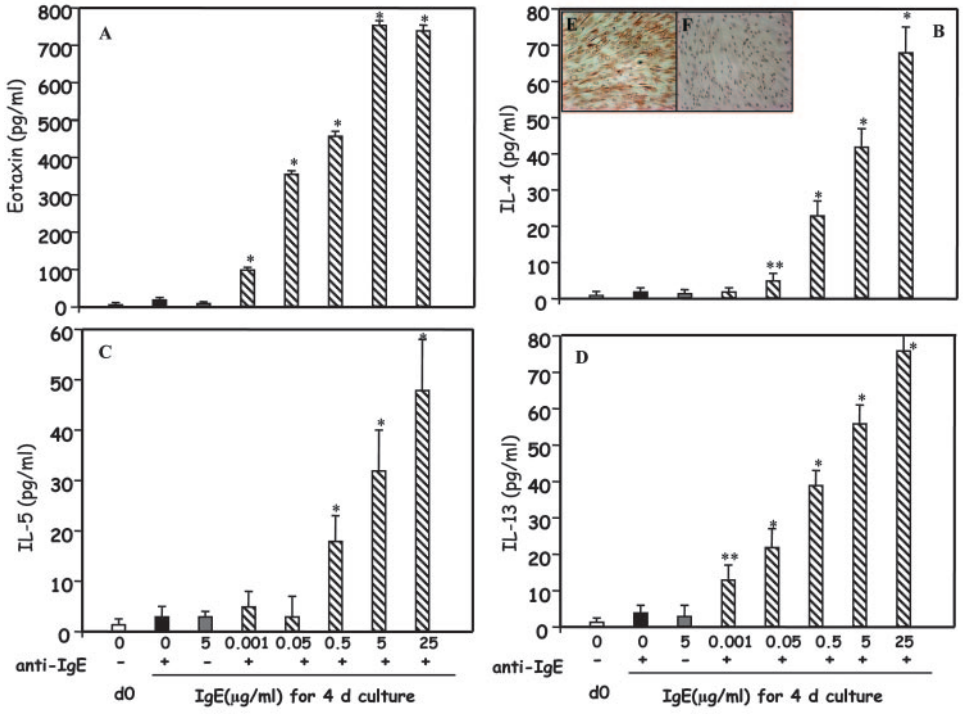

FIGURE 6. Effect of IgE stimulation on IL-4, IL-5, IL-13, and eotaxin protein release by B/TSM cells. B/TSM cells were sensitized first for 4 days with increasing concentrations of IgE, then sensitized for 30 min with IgE and challenged with anti-IgE for 12 h. Supernatants were recovered and clarified, and Th2 cytokines and eotaxin were measured by ELISA. Each point represents the mean ± SD of four measurements. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

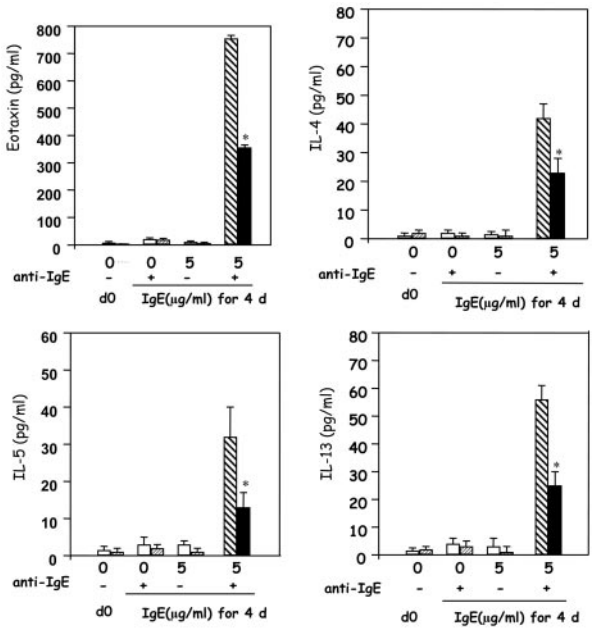

FIGURE 7. Neutralizing anti-FcεRI mAb abrogates IL-4, IL-5, IL-13, and eotaxin release from B/SM cells. Cells were cultured with IgE as described in Fig. 6 and anti-FcεRI mAb15–1, or mAb control was then added for 1 h before sensitization with IgE and challenge with goat anti-human IgE. These results represent mean ± SD of three independent experiments performed under the same conditions. Mann-Whitney *U* test was performed to analyze the difference between the samples. The significance of the difference is *, $p < 0.001$ compared with all other samples; and $p < 0.05$ compared with isotype mAb control.

28), it is tempting to speculate that local IgE could enhance FcεRI expression on ASM cells, which in turn upon cross-linking with the specific Ag leads to Th2 cytokines and chemokines release. Indeed, preliminary data from our laboratory revealed that incubation of ASM cells with various IgE concentrations results in FcεRI α-chain mRNA up-regulation (data not shown). Furthermore, Th2 cytokines, particularly IL-4, have been shown to play a positive role on the transcription of FcεRI α-chain in human mast cells (29, 30), human eosinophils obtained from atopic dermatitis subjects (31), and in dendritic cells (32). Whether a similar effect occurs in ASM cells is unknown. Given the invasive nature of the biopsy procedure, ethical considerations preclude us from obtaining ASM from bronchial biopsies from larger groups of asthmatic patients and normal controls to compare FcεRI expression in both groups. Further studies are under way to investigate the regulation of FcεRI expression in human ASM cells.

Activation of FcεRI triggers many signaling pathways in inflammatory cells including phosphorylation of FcεRI β and γ by Lyn, and the activation of Syk through its recruitment to FcεRI (19). Activation of Syk is crucial for FcεRI downstream signals including phosphorylation of phospholipase Cγ, calcium mobilization, and degranulation (19). In this study, we found that FcεRI activation leads to marked transient increases in intracellular Ca^{2+} concentration in human ASM cells, an effect that is likely a critical determinant of airway hyperresponsiveness through regulation of muscle contraction/relaxation (5). Contraction of smooth muscle is triggered by an increase in intracellular Ca^{2+} concentration in response to bronchospastic stimuli. For most contractile agonists, the increase in cytosolic Ca^{2+} is caused by mobilization of free Ca^{2+} from intracellular stores, as the result of binding of inositol 1,4,5-triphosphate to receptors on sarcoplasmic reticulum, which is followed by influx of Ca^{2+} from the extracellular milieu (33). Whether calcium mobilization after FcεRI engagement in ASM cells may rely on the same pathway as observed for contractile agonists is not known at this time. Further studies are required to establish which signaling pathways are involved in FcεRI activation in ASM cells, and whether this pathway influences ASM cell contraction.

Induction and perpetuation of airway inflammation involves a complex and coordinate response of multiple inflammatory cells, mediators, and cytokines. Recent evidence from our studies and others suggest that ASM is a potential source of multiple proinflammatory cytokines and chemokines (6, 34). In this study, we provide clear evidence that FcεRI-dependent activation of ASM cells induce IL-5 and eotaxin release. It is currently accepted that the allergen-induced eosinophil accumulation within lungs of atopic asthmatics is attributed to mature eosinophil migration from the circulation (35). Eotaxin was demonstrated as highly selective chemoattractant for eosinophils, basophils, and Th2-like T lymphocytes. It cooperates with IL-5 in vivo to induce eosinophil recruitment; IL-5 promotes mobilization of eosinophils from the bone marrow, whereas eotaxin recruits eosinophils in the tissue. Moreover, eotaxin has the ability to induce mast cell growth and eosinophil differentiation (36). Eotaxin is highly expressed by epithelial cells and inflammatory cells in asthmatic airways and has been found at high levels in bronchoalveolar lavage (BAL) fluid obtained from asthmatic subjects (37). We have shown recently that human ASM cells expressed eotaxin mRNA and protein following TNF-α and/or IL-1β stimulation (13). Eotaxin produced and secreted by ASM cells may amplify the chemokine signal generated by the infiltrating inflammatory cells in the airway, thereby augmenting the recruitment of eosinophils, basophils, and Th2 lymphocytes to the airways. The accumulation of these inflammatory cells may subsequently contribute to the development of airway hyperresponsiveness, local inflammation, and tissue injury through the release of granular enzymes and other cytokines. These growth factors may also induce proliferation of fibroblasts and smooth muscle cells in vitro, possibly leading to the observed increase in smooth muscle mass in the asthmatic airways. Similarly, cumulative data have suggested that IL-5 is the most important cytokine for the terminal differentiation of the committed eosinophil precursors and is a potent inducer of eosinophil survival (38). In the presence of IL-5, which delays the apoptotic process (39), eosinophils may persist within bronchial mucosa and cause further damage to the airways. As such, FcεRI-dependent activation of ASM cells may contribute to exaggerated airway eosinophilia through the recruitment of eosinophils and enhanced survival of infiltrating eosinophils.

In this study, we also found that ASM cells activated via FcεRI-dependent pathway induce the release of IL-4 and IL-13 but not IFN-γ. IL-4 and IL-13 have a number of actions relevant to allergic diathesis, such as the regulation of isotype class switching in B cell to IgE synthesis (40), induction of adhesion molecule expression on endothelial cells (41) promoting selective egress of eosinophils from the bloodstream (42, 43). Furthermore, recent studies have shown that both cytokines play a critical role in allergen-induced mucus production. In experimental models of allergic asthma (44, 45), selective neutralization of IL-13 ameliorated asthma symptoms, including airway hyperresponsiveness, eosinophil recruitment, and mucus overproduction (45). Recently, it was demonstrated that sensitized ASM cells could express Th1-type cytokines (IL-2, IL-12, and IFN-γ) hours after the initial up-regulation of Th2-type cytokines (46). It was suggested that ASM cell-derived IL-2 and IFN-γ may play a protective role in the airway, whereby exogenous IL-2 or IFN-γ attenuated atopic serum-induced ASM cell hyperresponsiveness to acetylcholine. IFN-γ

may also play a protective role in atopic asthma by functionally antagonizing IL-4-driven Ig isotype switching to IgE synthesis and Th1 cytokines may also be considered protective in asthmatic airways. This observation supports the notion that the proasthmatic state reflects an imbalance between Th1-type and Th2-type cytokine production. In accordance with these studies, transgenic overexpression of IL-4 and IL-13 within the lung in mice is associated with key features of airway inflammation and remodeling in chronic severe asthma, including lymphocyte and eosinophil accumulation, mucus cell metaplasia, subepithelial fibrosis, and airway hyperresponsiveness (47). In the light of the findings described above, the release of IL-4 and IL-13 after IgE-dependent activation of ASM cells via FcεRI, in combination with the eotaxin and IL-5 release may not only accentuate airway inflammation but may also participate in airway hyperresponsiveness. Furthermore, low levels of IFN-γ have been detected in the BAL fluid of patients with stable asthma, whereas the levels of mRNA for IFN-γ were not elevated in BAL fluid from patients with mild asthma (48). Our immunostaining results show also eotaxin and Th2 cytokine expression in the ASM cells obtained from asthmatic patients and provides a possible link between activation of ASM cells via FcεRI and airway inflammation and hyperresponsiveness.

Recently, Hirst et al. (49) have demonstrated that IL-13 and IL-4 induce selectively eotaxin release from ASM cells. It is tempting to speculate that IgE binding to the high affinity IgE receptor FcεRI induce IL-4 and IL-13 release, which in turn by an autocrine pathway leads to eotaxin and IL-5 release from ASM cells. Other possibilities cannot be ruled out because a recent study using mice deficient in both eotaxin and IL-5 has implicated both factors not only in tissue accumulation of eosinophil but also the ability of Ag-specific $CD4^+$ T cells to produce IL-13 and precluded the development of airway hyperresponsiveness (50). In light of the above studies, FcεRI activation of ASM cells may not only contribute to eosinophil migration, but also modulates Th2 cell production and subsequently airway hyperresponsiveness.

In conclusion, we have demonstrated for the first time the expression of functional high affinity IgE receptor FcεRI on ASM cells. This study suggests that IgE-dependent activation via the high affinity IgE receptor FcεRI could participate in airway inflammation, particularly eosinophilia by promoting cell recruitment and survival. It also suggests that ASM cell activation via FcεRI lead to the production of IL-4 and IL-13 that are involved in airway hyperresponsiveness, and intracellular calcium mobilization. Further studies are underway to clarify the signaling pathways involved in FcεRI-mediated smooth muscle activation.

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Disclosures

The authors have no financial conflict of interest.

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