Increased secretion of leukemia inhibitory factor by immature airway smooth muscle cells enhances intracellular signaling and airway contractility

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Fayon, Michael, Muriel Rebola, Patrick Berger, Sophie Daburon, Olga Ousova, Frédéric Lavrand, Bouthi Moukaïla, Wilfried Pujol, Jean Luc Taupin, André Labbé, Mathieu Molimard, and Roger Marthan. Increased secretion of leukemia inhibitory factor by immature airway smooth muscle cells enhances intracellular signaling and airway contractility. Am J Physiol Lung Cell Mol Physiol 291: L244–L251, 2006. First published February 17, 2006; doi:10.1152/ajplung.00474.2005.—Airway smooth muscle cells (ASMC) play a major role in airway inflammation, hyperresponsiveness, and obstruction in asthma. However, very little is known regarding the relation between inflammatory mediators and cytokines and immature ASMC. The aim of this study was to evaluate 1) the secretion of leukemia inhibitory factor (LIF) (an IL-6 family neurotrophic cytokine) by ASMC; 2) intracellular calcium concentration ([Ca2+]i) signaling; and 3) the effect of LIF on mast cell chemotaxis and rat airway contractility. Immature and adult human ASMC were cultured. ELISA and real-time PCR were performed to assess LIF protein secretion and mRNA production, [methyl-3H]thymidine incorporation to quantify ASMC DNA synthesis, a Boyden chamber to evaluate the effect of LIF on mast cell chemotaxis, microspectrofluorimetry using indo-1 (at baseline and after stimulation bradykinin, ACh) to evaluate the effect of LIF on intracellular signaling, and isolated rat pup tracheae to determine the effect of LIF on airway contractility to ACh. TNF-α-stimulated immature ASMC produce more LIF mRNA and protein than adult ASMC, although this cytokine induces a moderate increase in DNA synthesis (+20%) in adult ASMC only. Human recombinant LIF exerts no chemotactic effect on human mast cells. In immature ASMC, ACh-induced [Ca2+]i response was enhanced twofold after incubation with LIF, whereas TNF-α increased the [Ca2+]i to U-46619 threefold. In TNF-α-exposed adult ASMC, [Ca2+]i responses to ACh were of greater magnitude (sixfold increase) than in immature ASMC. Human recombinant LIF increased contractility to ACh by 50% in immature, isolated rat tracheae. Stimulated immature human ASMC greatly secrete LIF; thus potentially contributing to neuroimmune airway inflammation and subsequent remodeling. Increased LIF secretion enhances airway reactivity and [Ca2+]i signaling, calcium signaling, bronchial hyperreactivity, asthma.

Asthma very often begins in childhood and encompasses airway inflammation, obstruction, and hyperreactivity. It is now widely accepted that airway smooth muscle (ASM) cells (ASMC) possess new functions, in addition to their classical role as end-effector cells mediating bronchomotor tone. These functions include the secretion of cytokines and matrix proteins, cytokine-induced expression of cell adhesion molecule migration, proliferation, and cell-to-cell interactions by direct interaction with immune cells (8, 17, 25). An integral part of this interaction is the release of a variety of cytokines that regulate cellular and molecular responses. Indeed, several cytokines at the mRNA or protein level have been detected within the airways of asthmatic subjects (6). These proinflammatory mediators may act directly or indirectly, in an autocrine manner, on ASMC. All of the above greatly contribute to the perpetuation of the chronic local inflammation and the structural remodeling process of asthmatic airways. This complex inflammatory response to injury may also involve the coordinated interaction between the nervous and immune systems (27). Leukemia inhibitory factor (LIF), a member of the IL-6 family of cytokines, has also been shown to be an integral component of the interface between nerves and the immune system (19). However, little is known about this cytokine in the context of normal lung function or indeed, inflammation, especially in immature humans and animals.

The asthmatic inflammatory process may influence calcium signaling. The study of Ca2+ signaling provides information regarding ASMC contractile activity (22). Stimulation of an ASMC by a bronchoconstrictor agent induces a rapid rise in intracellular Ca2+ concentration ([Ca2+]i), mostly related to the release of intracellular Ca2+ stores, to a peak level roughly 10 times higher than the resting level (2). The binding of ACh to a muscarinic M3 cholinoreceptor activates a Gq/11 protein that, in turn, activates phospholipase C, inducing hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce inositol 1,4,5-trisphosphate and 1,2-diacylglycerol. Inositol 1,4,5-trisphosphate binds to its receptor to release Ca2+ from the sarcoplasmic reticulum (34). The increased [Ca2+]i, results in the activation of the Ca2+/calmodulin-sensitive myosin light-chain kinase, and the subsequent phosphorylation of the regulatory myosin light chain, which results in the cross bridging between myosin and actin (2). The ability of TNF-α to alter the [Ca2+]i, signals induced by a variety of agonists suggests that this cytokine may

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prime ASMC, with subsequent hyperresponsiveness to contractile agents (2).

Despite the extensive literature, very little is known of the intrinsic properties of immature ASM. It is thus important to specifically characterize the in vitro properties of the immature ASM in humans. Since epidemiological studies in asthma suggest that outcome in adults may be determined primarily in early childhood (23, 24, 36), we hypothesized that immature ASM may contribute more toward the inflammatory process and airway remodeling than adult ASM. The aim of the present study was thus to evaluate the secretory properties of cultured human neonatal ASM (secretion of the neurotrophic cytokine LIF and production of its mRNA). Moreover, we studied the effect of LIF on DNA synthesis in ASM, migration of human mast cells (HMCs), intracellular ASM [Ca\(^{2+}\)], signaling, and contraction of isolated rat pup tracheae. Moreover, the effect of other inflammatory mediators (histamine, U-46619, bradykinin) and contractile agonists (ACH) on [Ca\(^{2+}\)], was also evaluated. The findings were compared with adult cells.

MATERIALS AND METHODS

**Human ASM**

Isolation and culture of human neonatal and adult ASMCs. Human lung was obtained by postmortem forceps biopsy from premature (≥26 wk) and term neonates and infants who had died from extrapulmonary causes, and at thoracotomy in adults undergoing resection for pulmonary carcinoma, as previously described (4, 12). The time between the death of the neonates and infants and harvesting of the tissue was <2 h in all cases. Adult patients were nonatopic and did not report any clinical history of asthma. The tissue obtained (trachea in neonates, and 3rd to 5th generation airways in adults) was used for cell cultures. Collection and use of lung specimens were in accordance with the ethics recommendations of Bordeaux University Hospital and French law, and our laboratory, which is authorized to conduct human and animal studies, obtained informed consent from the parents.

The trachea and bronchi were washed three times with sterile DMEM containing 100 µg/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (antimycotic-antibiotic solution; GIBCO). Segments of airways were dissected free from the surrounding parenchyma in the same medium at room temperature. The epithelium was removed to expose the underlying bands of smooth muscle, which were then gently separated from the underlying connective tissue in small bundles. Smooth muscle bands were cut into squares measuring 1–2 mm\(^2\) and transferred into six-well culture plates (∼1–2 explants/well) and covered with a minimal amount of medium and 10% (vol/vol) FCS (GIBCO), supplemented with 2 mM L-glutamine (GIBCO), 1 mM sodium pyruvate (Sigma Chemical), 1% (vol/vol) nonessential amino acid mixture (Sigma Chemical), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (antimycotic-antibiotic solution; GIBCO). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO\(_2\)-95% air. The medium was replenished every 3 days. Cells were passaged once they reached confluence (after 4–8 wk), and passages 2–5 were used for experimentation. Cells were rinsed twice with HBSS (GIBCO) and then passaged with trypsin-EDTA (GIBCO).

Cell characterization by immunocytochemistry. To assess the purity of the cells, an immunocytochemical method was employed using an indirect immunofluorescence technique (4). Briefly, after fixation of ASMC in methanol, anti-α actine (clone 1A4, Sigma), anti-myosin (clone hSM-V, Sigma), anti-epithelial cell cytokeratin (Sigma), anti-endothelial cell factor VIII (Dako), and anti-fibroblast cellular surface protein (clone 1B10 Sigma) primary antibodies were used to confirm that the cells were ASMC. All human ASMCs stained positively for smooth muscle actin and myosin. An irrelevant antibody served as a control.

Analysis of LIF levels in ASMC culture supernatant. To assess whether the ASMC secreted LIF, TNF-α (100 ng/ml) was added to the medium after the cells had been starved in insulin, transferrin, and selenium (ITS) medium for 24 h. The supernatant was then harvested every day thereafter (days 1–5), stocked on ice, and immediately frozen (−80°C). The LIF assay was performed as previously described (29) using a colorimetric ELISA. Briefly, specific high-titer polyclonal Ig were obtained by intra-lymph node immunization of rabbits with recombinant human (rh) LIF. The same batch of purified anti-LIF Ig was used for the coating (capture antibody) and for the quantitative detection of the bound cytokine (soluble biotinylated antibodies). The sensitivity of this ELISA is 12 pg/ml of LIF (28).

RNA extraction and real-time PCR. We extracted total RNA from cultured ASMC, 600 µl of Trizol, according to the manufacturer’s instructions (GIBCO, Invitrogen, Cergy Pontoise, France). RNA integrity (the integrity of the 28S and 18S fractions of rRNA) was verified by gel electrophoresis. The concentration of total RNA was measured by GeneQuant RNA/DNA calculator (Amersham Pharmacia). The reverse transcription reaction was carried out as mentioned previously (4). Real-time PCR was performed with a Rotor-Gene 2000 (Corbett Research; Morlake, Sidney, Australia) as described earlier (4). All specific primers were designed by using oligo primer analysis software (Oligo 6.6, Molecular Biology Insights, Cascade, CO) and ordered from Sigma-Genosys (Sigma-Genosys, Cambridge, UK). The primer pair sequences are indicated in Table 1. The efficiency of all of the PCR reactions was >90%. The specificity of the amplified products was examined in 2% agarose gel containing ethidium bromide. The accuracy of the normalization of real-time PCR data was verified by geometric averaging of four internal control genes (see above), according to a previously described method (38).

DNA synthesis assay. Once harvested by trypsinization, cells were grown in 96-well plates (2,000 cells/well), starved for 24 h (serum-free DMEM supplemented with 10 µg/ml insulin, 5.5 µg/ml transferrin, 5 ng/ml selenium, 0.5 µg/ml BSA, 4.7 µg/ml linoleic, and oleic acid) (ITS solution, Sigma Chemical), 2 mM L-glutamine (GIBCO), 1

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Table 1. **Specific primer-pairs used for gene-expression analysis by real-time reverse transcription PCR**

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<th>Primer</th>
<th>GenBank Accession No.</th>
<th>Sequence</th>
<th>Product, bp</th>
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<td>252</td>
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<td></td>
<td></td>
<td>reverse 5’-GGA GGT GCC AAG GTA CAC GAC-3’</td>
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<tr>
<td>GAPDH</td>
<td>NM_002046</td>
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<td>108</td>
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<td></td>
<td></td>
<td>reverse 5’-AGCGAATGTTGTTGCATACG-3’</td>
<td></td>
</tr>
<tr>
<td>PRLPO</td>
<td>NM_001002</td>
<td>forward 5’-CAACGGGTACCAACGACTC-3’</td>
<td>182</td>
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<tr>
<td></td>
<td></td>
<td>reverse 5’-CTCCGGGTTGTACCAACCTTAC-3’</td>
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<td></td>
<td></td>
<td>reverse 5’-ATGGAAGGGAGATATCCTCTACAAA-3’</td>
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Target genes: huLIF, human leukemia inhibitory factor. Control genes: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PRLPO, ribosomal protein, large, PO; HPT1, hypoxanthine phosphoribosyl transferase 1.
mM sodium pyruvate (Sigma Chemical), 1% (vol/vol) nonessential amino acid mixture (Sigma Chemical), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (antimycotic-antibiotic solution, Gibco), and incubated for 1–5 days with medium, 10% FCS, ITS, or PDGF-AA (15 ng/ml diluted in ITS medium). After the incubation period, 0.5 mCi [methyl-3H]thymidine (Amersham) was added to each well for an additional 8 h. Cell proliferation was then arrested by placing the culture plates in a 20°C freezer. Cells were harvested on a 0.7-mm-pore glass fiber filter and counted in scintillant on a 2000CA TriCarb liquid scintillation analyzer (4).

Chemotaxis assay. The migration of HMC-1 cells against recombinant LIF was assayed by using a 48-well micro-chemotaxis (Boyden) chamber (Neuroprobe, Cabin John, MD), as described previously (4). Nitrocellulose filters (150-μm thick, pore size of 8 μm) (Millipore, St. Quentin en Yvelines, France) were coated overnight at room temperature with 100 μg/ml fibronectin (Sigma) and then air-dried for 30 min. Migration of HMC-1 cells (3 × 104 cells/ml) was assessed in response to 0.2, 2, 20, and 200 ng/ml rhLIF (Chemicon International). Each experimental condition was processed in triplicate. After 60 min at 37°C and 5% CO2, nonmigrant cells were removed from the upper surface, and the filter was then fixed in ethanol, stained with Harris hematoxylun, and mounted in Permount (Fisher, Elancourt, France). The number of migrant cells present in the whole surface of each well (i.e., 8 mm2) was counted by using a quantitative method of color recognition described previously (5). The spontaneous migration of mast cells with either supernatant from unstimulated human ASMCs or DMEM served as controls for human ASMC supernatant or cytokine chemotaxis, respectively, and was referred to as 100% migration. Control experiments showed that mast cells that had migrated through the filter were not communal, as no cell was detected in the fluid phase of the lower chamber. In addition, chemokinesis was differentiated from chemotaxis by placing attractants at the same concentrations in the upper wells.

Intracellular [Ca2+]i signaling. Once harvested by trypsinization, cells were placed on 14-mm circular glass plates and starved for 3 days in ITS medium. This medium was supplemented during the last 48 h (stimulated cells) or not (control cells) with the proinflammatory cytokine TNF-α (100 ng/ml) or LIF (5 ng/ml). Cells were then rinsed and incubated for 30 min at room temperature in a physiological saline solution with 2 mM Ca2+ (composition in mM: NaCl 130; KCl 5.6; CaCl2; 2 CaCl2; 10 HEPES; 11.1 glucose; 1 MgCl2) containing 1 μM of esterified indo-1 (indo-1 AM). Four different contractile agonists (histamine, bradykinin, ACh, and U-46619, a thromboxane A2 agonist) were used. 

DNA Synthesis

Cultured cells assayed 1 and 5 days in ITS medium showed significant levels of incorporation of [methyl-3H]thymidine: day 1: 440.9 ± 147.4 vs. 454.1 ± 49.9; day 5: 449.1 ± 89.4 vs. 387.6 ± 81.0 in immature (n = 5) and adult (n = 5) ASMC, respectively (P = 0.037). At all study times, LIF secretion was enhanced after the addition of TNF-α (100 ng/ml) to the medium (Fig. 1). There was a 14-fold increase by adult ASMC and 24-fold increase by immature ASMC after 5 days of stimulation by the proinflammatory cytokine. In one case in immature ASMC, LIF secretion was increased by more than a power of 102.

LIF mRNA Expression

In immature ASMC stimulated by TNF-α (100 ng/ml), LIF mRNA was upregulated in a sustained manner, i.e., 3–24 h after the addition of TNF-α (n = 3). In contrast, adult ASMC mRNA levels decreased from hour 3 to 24 (n = 5) (Fig. 2).

Migration of HMC-1 Cells

The effect of exogeneous rhLIF alone on migration of HMC-1 cells was negligible (% values in ITS medium): 109.2 ± 1.0, 103.0 ± 11.2, 104.5 ± 14.1, and 105.9 ± 6.2% at a LIF concentration of 0.2, 2, 20, and 200 ng/ml, respectively (n = 4 per concentration).

In LIF. Incubation of immature ASMC in 5 ng/ml LIF greatly enhanced [Ca2+]i; response to ACh, compared with control conditions (ITS medium) (Fig. 3). Baseline [Ca2+]i, increased 59% [317.8 ± 26.5 nM (n = 18) vs. 505.8 ± 33.9
nM (n = 24) in ITS medium and LIF, respectively (P < 0.001). Relative [Ca\(^{2+}\)], peak increased more than twofold (248%) from 210.4 ± 22.9 to 522.0 ± 43.6 nM (P < 0.001), and time to maximal peak decreased twofold from 5.19 ± 0.51 to 2.47 ± 0.18 s (P < 0.001). There was a significant difference in baseline [Ca\(^{2+}\)], and time to maximal [Ca\(^{2+}\)] peak after ACh stimulation in immature and adult ASMC (100 ± 13 nM and 14.8 ± 0.8 s, respectively) (Fig. 3).

After incubation with TNF-α. After 3 days of incubation with the proinflammatory cytokine TNF-α, immature cells showed a trend toward greater relative [Ca\(^{2+}\)], peak after stimulation by the four contractile agonists studied compared with control cells (Fig. 4). However, the enhanced response reached statistical significance after stimulation by U-46619 only (184.8 ± 66.6 vs. 66.2 ± 15.4 nM, n = 15 per agonist, P < 0.01, in stimulated and control cells, respectively). Mature cells responded significantly to ACh only (400.8 ± 122.1 vs. 64.5 ± 26.3 nM, n = 15 per agonist, P < 0.01). This response in adults was much more marked compared with controls (sixfold increase) than in immature cells (twofold increase).

**Contractility of Rat Pup Isolated Tracheae**

As shown in Fig. 5, there was a 48% increase in maximal isometric force generated by 10\(^{-3}\) M ACh after stimulation by LIF [1,391 ± 159 mg (LIF, n = 11) vs. 941 ± 177 mg (control group, n = 10); P < 0.05].
DISCUSSION

In the present study, we have shown that TNF-α-stimulated immature ASMC massively produce LIF mRNA and protein, although this proinflammatory neurotrophic cytokine moderately increases DNA synthesis in adult ASMC only and has no relevant chemotactic effect on HMCs. The main demonstrable effect of LIF involved Ca²⁺ signaling and isometric airway contractility to ACh in immature, isolated rat trachea. The proinflammatory cytokine TNF-α did not significantly enhance Ca²⁺ signaling to ACh in immature ASMC, as opposed to mature cells. However, [Ca²⁺]ᵢ was greatly enhanced in immature cells after stimulation by the thromboxane analog U-466619. This work, together with our own previous work, thus suggests that immature ASMC are already in a primed state and that increased LIF secretion may be one of the triggers of this chain reaction.

The ASMC is at the center of a dynamic asthmatic response and plays an important role in the hyperresponsiveness and remodeling that occur in the asthmatic airway, by subserving inflammation-driven fibro-proliferative remodeling of the airway wall, including increased muscle mass, matrix protein deposition in the muscle layer, submucosa, and adventitial (16). This results from its contractile and migratory properties, as well as its active participation in the inflammatory response via the autocrine production of cytokines and chemokines (37). The IL-6 family of cytokines, which includes IL-6 itself, LIF, IL-11, oncostatin M, cardiotropin 1, and ciliary neutrophic factor, as well as other cytokines, plays an important role in airway inflammation (11). As an example, oncostatin M causes eotaxin-1 release from ASM, in synergy with IL-4 and IL-13, by a mechanism involving STAT-3 (11). To the best of our knowledge, the secretion of LIF by human neonatal ASMCs has not been studied. In the present study, we have demonstrated that cytokine is massively released and may enhance the inflammatory response. Our investigations into the deleterious effect of exogenous LIF showed that it greatly increases airway signaling and contractility to the cholinergic agonist ACh.

Ca²⁺ plays a central role in the activation of many cellular processes, including the most relevant end point in ASM physiology: contraction. For this reason, the cytosolic concentration of Ca²⁺ is tightly controlled. Ca²⁺ mobilization and signaling is a postreceptor event that is closely related to the muscarinic neural system and phosphoinositide hydrolysis. It may be induced by contractile agonists, whose effect is also modulated by the ASMC inflammatory microenvironment. Indeed, when stimulated by Th2-type cytokines, airways adopt a proasthmatic phenotype of altered hyperresponsiveness (15). The autocrine-induced changes in ASM responsiveness are attributed to altered receptor-coupled transmembrane signaling in the sensitized ASM, resulting in perturbed expression and release of second-messenger molecules that regulate ASM contraction and relaxation (15). Cyclic ADP-ribose (cADPr), Fig. 3. Intracellular calcium concentration ([Ca²⁺]ᵢ) after 2 days of incubation with or without LIF. Results are means ± SE. ASMC were incubated for 48 h in ITS medium alone (immature, n = 18; mature, n = 53) or with LIF (5 ng/ml; immature, n = 24). They were then stimulated by 10⁻⁵ M (adult) or 10⁻⁴ M (immature) ACh. A: representative [Ca²⁺]ᵢ response curve in immature ASMC in ITS medium (dotted line) or LIF (solid line). B: baseline (left), relative peak (middle), and time to peak (right) [Ca²⁺]ᵢ responses. *P < 0.001 (ANOVA).
an endogenous activator of the ryanodine receptor channel in mammalian cells, has been shown to modulate agonist-induced Ca$^{2+}$ responses in ASM cells (10, 39). In addition, cADPr-mediated Ca$^{2+}$ release appears to play an important role in the “nonspecific” increased ASM responsiveness to contractile agonists in cytokine-treated cells, a characteristic finding of asthma (10, 39). Furthermore, other signaling molecules, such as Rho/Rho kinase and phosphodiesterase, also contribute to bronchial hyperresponsiveness (3). In the present study, we have demonstrated that LIF enhances baseline and relative peak [Ca$^{2+}$]i response to ACh in moderately serum-deprived immature ASMC (cells that are reacquiring their contractile protein content). This response is significantly greater than what is observed in adult ASMC. The mechanisms involved in this increased [Ca$^{2+}$]i response to LIF require further studies.

However, a number of studies have investigated the role of the proinflammatory cytokine TNF-α on ASMC contraction. TNF-α may act directly by altering the G-protein-mediated signal transduction (activation of pathways dependent on protein synthesis) (2, 3, 18), or the intracellular Ca$^{2+}$ pumps (decreased sequestering of Ca$^{2+}$ into intracellular stores by interfering with the sarco-endoplasmic reticulum Ca$^{2+}$-ATPases), rather than by modifying the expression of contractile agonist receptors (2). The inflammatory process may render ASMC hyperresponsive to contractile agents, which might be, at least in part, mediated through activation of the Ras/Raf/MEK/MAPK pathway (40). In another study, it has been shown that the augmentation of ACh-induced contractile response is mediated, at least in part, by synthesis of protein, such as RhoA, through activation of p42/44, but not p38 MAPK (35). Additionally, TNF-α caused significant augmentation of CD38 expression (a cell surface protein that catalyzes the synthesis and degradation of cADPr), ADP-ribosyl cyclase activity, and Ca$^{2+}$ responses to ACh, bradykinin, and thrombin (10). In the present study, TNF-α significantly increased the [Ca$^{2+}$]i response to U-46619 in immature ASMC and showed a trend toward a greater response when stimulated by the other inflammatory mediators such as bradykinin. Adult ASMC also showed a marked response to ACh after being primed by TNF-α. TNF-α is an interesting candidate mediator in airway inflammatory diseases, considering that high levels of this substance are found in asthmatic airways (1). It results from infiltration of the airways by mast cells, monocytes, and epithelial cells (7). Importantly, the number of mast cells within the ASM bundles themselves in an asthmatic airway is increased (4). This would potentially result in a greater concentration of TNF-α within the vicinity of the ASM bundle. TNF-α has thus the potential to modulate the interactions between cells present in the inflamed airways of a patient with asthma and, therefore, to contribute to the regulation of airway inflammation and remodeling (7).
Similarly, hyperresponsiveness to ACh after incubation with LIF has been demonstrated in the present study in an isolated airway rat model. This is compatible with the findings regarding human ASM Ca\(^{2+}\) homeostasis, although the experimental conditions are not entirely comparable (Ca\(^{2+}\) measurements were carried out in cells following incubation with LIF for 48 h, while contractility of tracheal rings was measured following 24-h incubation). The interaction between LIF and the cholinergic system suggests a role for LIF in the regulation of neurotransmitter and neurotransmitter receptor phenotype, particularly muscarinic receptors. LIF is an integral component of the interface between nerves and the immune system (19). Evidence is emerging that this cytokine may also play an important role in regulating the neural-immune system interaction during acute inflammatory insult and the subsequent healing and restitution process. In guinea pig airways, it amplifies the contractile response to tachykinins (20). LIF may thus act as a mediator of bidirectional cross talk between neural tissue and the immune system (21). This is of importance, since convincing experimental work in a rat model has highlighted the importance of neurogenic inflammation in acute viral bronchiolitis and its role in subsequent inflammation and reactive airways disease, which may lead to asthma (26).

There is also increasing evidence to support the hypothesis that LIF and related cytokines play both direct and indirect roles in the proliferative response of epithelial and mesenchymal cells (19). In our study, however, the effect of LIF on ASM DNA synthesis and mast cell migration was very modest. This may be due to the fact that LIF does not induce STAT-3-dependent gene transcription (11). An indirect action via the potentiation of muscarinic M\(_3\)-receptor stimulation is also a possibility. Noteworthy, muscarinic stimulation enhances ASM proliferation to peptide growth factors in vitro (13). It is also likely that the deleterious effect of LIF on ASM function may be potentiated by the combined presence of other mediators, growth factors, or cytokines (11). For example, in eosinophils, LIF does not appear to influence chemotaxis per se, although it markedly upregulates eosinophil migration in response to classical agonists such as platelet-activating factor, as well as substance P (19). Similarly, the PDGF-dependent proliferation of nontransformed human fibroblasts, vascular smooth muscle cells, and mesangial cells involves the action of IL-6 (32). Once released, LIF may exert a number of autocrine and paracrine effects.

The fact that immature ASM exhibits enhanced responses to inflammatory mediators and cytokines, albeit in a different manner compared with adults, may have implications for the early life environment. Numerous studies have shown the potential influence of in utero cytokine levels on the risk of atopy (30, 31). It is also known that the cytokine-driven autocrine signaling mechanism in ASM may be triggered by either Fc-receptor activation in the atopic (IgE-mediated) sensitized state or by ASM exposure to specific viral respiratory pathogens, most notably including rhinovirus (14). Rhinovirus 16 elicits proinflammatory changes in ASM responsiveness that are attributed solely to binding of the virus to its host receptor (ICAM-1) on the ASM cell surface (14). The present study adds further evidence to the risk of early increased airway hyperresponsiveness and remodeling in the presence of a proinflammatory ambiance.

The main limitation of the study lies in the fact that this is not a true ontogenetic study, since only two age groups were studied: neonates and adults. This precludes the extrapolation of our results to infants and preschool children for whom we have the greatest gap in our knowledge. Moreover, the airway generations from which the smooth muscle was taken were different in the two age groups (cultured cells from tracheae in neonates vs. intrapulmonary airways in adults). This is due to the major difficulties in obtaining human lung tissue. The conclusions have thus to be interpreted with caution. Nonetheless, from the results, one can gain an insight into the properties of immature human ASM.

In conclusion, we supply evidence suggesting that human ASM significantly secretes LIF (immature \(\gg\) adults) upon stimulation by the proinflammatory cytokine TNF-\(\alpha\). Immature ASM exhibit increased [Ca\(^{2+}\)]\(_i\) responses to LIF and the thromboxane analog U-46619, as well as increased airway contractility to LIF, while Ca\(^{2+}\) signaling in mature ASM was more marked in the presence of ACh. Taken together, the data from our study indicate that immature ASM are primed to amplify inflammation in the airways.

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