

Role of nicotinic receptors and acetylcholine in mucous cell metaplasia, hyperplasia, and airway mucus formation *in vitro* and *in vivo*

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Background: Airway mucus hypersecretion is a key pathophysiologic feature in a number of lung diseases. Cigarette smoke/nicotine and allergens are strong stimulators of airway mucus; however, the mechanism of mucus modulation is unclear.

Objectives: We sought to characterize the pathway by which cigarette smoke/nicotine regulates airway mucus and identify agents that decrease airway mucus.

Methods: IL-13 and γ -aminobutyric acid type A receptors (GABA_ARs) are implicated in airway mucus. We examined the role of IL-13 and GABA_ARs in nicotine-induced mucus formation in normal human bronchial epithelial (NHBE) and A549 cells and secondhand cigarette smoke–induced, ovalbumin-induced, or both mucus formation *in vivo*.

Results: Nicotine promotes mucus formation in NHBE cells; however, the nicotine-induced mucus formation is independent of IL-13 but sensitive to the GABA_AR antagonist picrotoxin. Airway epithelial cells express $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ -nicotinic acetylcholine receptors (nAChRs), and specific inhibition or knockdown of $\alpha 7$ - but not $\alpha 9/\alpha 10$ -nAChRs abrogates mucus formation in response to nicotine and IL-13. Moreover, addition of acetylcholine or inhibition of its degradation increases mucus in NHBE cells. Nicotinic but not muscarinic receptor antagonists block allergen- or nicotine/cigarette smoke–induced airway mucus formation in NHBE cells, murine airways, or both.

Conclusions: Nicotine-induced airway mucus formation is independent of IL-13, and $\alpha 7$ -nAChRs are critical in airway mucous cell metaplasia/hyperplasia and mucus production in

response to various promucoid agents, including IL-13. In the absence of nicotine, acetylcholine might be the biological ligand for $\alpha 7$ -nAChRs to trigger airway mucus formation. $\alpha 7$ -nAChRs are downstream of IL-13 but upstream of GABA_AR $\alpha 2$ in the MUC5AC pathway. Acetylcholine and $\alpha 7$ -nAChRs might serve as therapeutic targets to control airway mucus. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Cigarette smoke, nicotine, nicotinic acetylcholine receptors, γ -aminobutyric acid receptors, acetylcholine, airway mucus

Normal mammalian airway epithelium produces and is coated by mucins, such as MUC5B and MUC5AC, and after stimulation by an allergen/infection, MUC5AC is the predominant mucin produced in human airways. These mucins assist in clearing inhaled particulate matter from the airways.¹ However, excessive mucous cell metaplasia and mucus hypersecretion contribute to the pathology of many respiratory diseases, such as chronic obstructive pulmonary disease, asthma, and cystic fibrosis.² In addition, excessive mucus production prolongs lung infections and decreases lung function.³ Cigarette smoke is a strong inducer of airway mucus production and a major risk factor for asthma, bronchitis, and chronic obstructive pulmonary disease.^{4,5} Moreover, recent studies suggest that nicotine promotes airway mucus formation^{6,7}; however, the mechanism by which cigarette smoke/nicotine promotes mucus formation is not well established. Studies have demonstrated that T_H2 cytokines, particularly IL-13, are key mediators of mucous cell metaplasia/hyperplasia and mucus production⁸⁻¹⁰; however, in a rat allergic asthma model, chronic nicotine treatment strongly downregulated IL-4 and IL-13 production but increased mucous cell metaplasia and mucus production in the lung.¹¹ Thus in this model of allergic asthma, nicotine might stimulate mucus formation independently or semi-independently of IL-13.

A number of nonneuronal cells, including T cells, macrophages, and lung epithelial cells, express nicotinic acetylcholine receptors (nAChRs) and might synthesize acetylcholine.^{12,13} Mucus-producing lung epithelial cells from rats, mice, and human subjects also express several different γ -aminobutyric acid type A receptor (GABA_AR) subunits, which have been implicated in airway mucus formation.¹⁴ Nicotine is a major constituent of cigarette smoke, and in the central nervous system nicotine activates GABA_ARs in some neurons. Moreover, the $\alpha 7/\alpha 9/\alpha 10$ -nAChR antagonist methyllycaconitine (MLA) moderates mucus formation in monkey lungs.⁶ We hypothesized that in airway epithelial

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Abbreviations used

AB/PAS:	Alcian blue/periodic acid–Schiff
ALI:	Air-liquid interface
GABA _A R:	γ-Aminobutyric acid type A receptor
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
IHC:	Immunohistochemistry
MLA:	Methyllycaconitine
MM:	Mecamylamine
nAChR:	Nicotinic acetylcholine receptor
NB:	Neostigmine bromide
NHBE:	Normal human bronchial epithelial
OVA:	Ovalbumin
PIC:	Picrotoxin
qPCR:	Quantitative PCR
siRNA:	Small interfering RNA
SS:	Secondhand smoke
TBST:	Tris-buffered saline containing 0.05% Tween-20
TCR:	T-cell receptor

cells nicotine activated GABA_ARs through nAChRs, thereby promoting mucus formation. In this communication we show that although normal human bronchial epithelial (NHBE) cells express the α7-, α9-, and α10-nAChR subunits, α7-nAChRs play a critical role in mucous cell metaplasia and mucus formation in NHBE cells. Moreover, (1) nicotine promotes mucus formation independently of IL-13, (2) the normal biological ligand for the nAChRs in the bronchial epithelial cells for mucus formation might be acetylcholine, and (3) antagonists of nAChRs but not muscarinic receptors suppress mucus formation *in vivo* and *in vitro*.

METHODS

NHBE and A549 cells were cultured by using standard procedures.¹⁵ DO11.10 ovalbumin (OVA)–T-cell receptor (TCR) transgenic mice on a BALB/c background were exposed to air, secondhand smoke (SS) or nicotine (1.5 mg total particulate material/m³), heat-aggregated OVA aerosol (5 mg/m³), or OVA plus SS or nicotine for 2 weeks (6 h/d for 5 d/wk). In some experiments normal (wild-type) BALB/c mice were sensitized to *Aspergillus fumigatus* extract, as described previously.¹⁶ Where indicated, mice were subcutaneously implanted with mecamylamine (MM)–containing miniosmotic pumps (2 mg/kg body weight per day) 3 weeks before exposure.¹⁷ In another group of BALB/c mice, animals were first exposed subcutaneously to saline (control)– or MM-containing ALZET pumps (DURECT Corp, Cupertino, Calif) for 2 weeks and then sensitized with *A. fumigatus* allergen extracts, as described in the **Methods** section in this article's Online Repository at www.jacionline.org. To determine the effects of nicotine, IL-13, or acetylcholine on NHBE or A549 cells, cells were treated with nicotine base (100 nmol/L), recombinant human IL-13 (10–50 ng/mL), or indicated concentrations of neostigmine bromide (NB), respectively, and the cultures were harvested approximately 48 hours later. GABA_AR, nAChR, or muscarinic receptor inhibitors were added at the indicated concentrations 2 hours before the addition of IL-13, nicotine, or NB. NHBE cells (5-μm-thick sections) were stained with Alcian blue/periodic acid–Schiff (AB/PAS) staining for mucus.^{18,19} MUC5AC, or GABA_ARα2 by using appropriate reagents, and the cell number and mucus volume were determined by using microscopy.^{11,20} Murine lung sections were stained for MUC5AC and GABA_ARα2 by using immunohistochemistry (IHC). Total RNA was isolated from lung tissues, NHBE cells, and A549 cells with TRI-Reagent (Molecular Research Center, Inc, Cincinnati, Ohio). GABA_ARα2-specific mRNA was assayed by using SuperScript III One-Step RT-PCR with Platinum Taq (Invitrogen, Carlsbad, Calif). RT-PCR primers for GABA_ARα2 were 5'-AGGCTTCCGTTATGATACAG (forward) and 5'-AGGACTGACCCCTAATACAG (reverse), and those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5'-CCCATCA

CCATCTTCCAGGAG (forward) and 5'-TTCACCACCTTCTTCTTGATGT CAT (reverse). Quantitative PCR (qPCR) was performed with a TaqMan One-Step RT-PCR kit containing AmpliTaq Gold DNA polymerase with ABI primers and probes. Fold differences were determined by using the 2^(-ΔΔCT) method.²¹ nAChR subtypes were knocked down by specific small interfering RNAs (siRNAs); changes in specific mRNAs were determined 48 hours after siRNA treatment. Protein levels of GABA_ARα2 were determined by means of Western blot analysis.¹⁷ GraphPad Prism Software 5.03 (GraphPad Software, Inc, La Jolla, Calif) was used to determine statistical significance by means of 2-way ANOVA. Detailed methods are given in the **Methods** section in this article's Online Repository.

RESULTS**Nicotinic receptors are critical in mucus formation**

We examined the effects of nicotine (100 nmol/L) on mucus formation in NHBE cells grown at the air-liquid interface (ALI). This is a realistic concentration of nicotine and is several-fold lower than the median effective concentration (10–100 μmol/L) of nicotine/nicotine agonists required to activate the ligand-gated cationic channel in neurons.²² As seen with AB/PAS mucus staining (Fig 1, A), control NHBE cells have a low baseline level of mucus; however, when the cells were treated with nicotine, IL-13, or IL-13 plus nicotine for 48 hours (predetermined optimal time), the mucus content in these cells increased strongly. Furthermore, the α7/α9/α10-nAChR-specific antagonist MLA (Fig 1, A), as well as the nonselective nAChR antagonist MM (Fig 1, B), suppressed the nicotine plus IL-13–induced mucus formation in NHBE cells. MLA also blocked the increase in mucus formation in NHBE cells in response to either nicotine or IL-13 (see Fig E1 in this article's Online Repository at www.jacionline.org). To determine whether nicotine, IL-13, or both affected mucous cell hyperplasia and metaplasia, we measured the number of mucous cells per millimeter of basal lamina and the volume of mucus-containing cells (mucus volume per cubic millimeter of basement membrane), respectively. Nicotine and IL-13 significantly increased both mucous cell numbers (Fig 1, C, left panel) and volume (Fig 1, C, right panel), and these effects were blocked by MLA. These results suggest that both nicotine and IL-13 affect mucous cell physiology and require the activation of nAChRs (α7, α9/α10, or both). Although in some experiments a combined treatment with nicotine and IL-13 appeared to increase cell volume over that seen after individual treatment with IL-13 or nicotine, these differences varied from experiment to experiment and were not statistically significant (data not shown). Thus nicotine and IL-13 might affect the same downstream pathway or pathways for mucous cell hyperplasia/metaplasia and mucus production.

Nicotine and IL-13 increase MUC5AC expression

Mucin glycoproteins are the major constituents of airway mucus. *MUC5AC* is the dominant mucin gene expressed in airway goblet cells,²³ and IL-13 is known to increase *MUC5AC* expression in these cells.²⁴ NHBE cells were treated with nicotine, IL-13, or both and *MUC5AC* expression was examined by means of qPCR and IHC staining with the *MUC5AC*-specific antibody to ascertain whether nicotine, IL-13, or both also induced the expression of *MUC5AC*. Both nicotine and IL-13 significantly increased the mRNA expression of *MUC5AC*; however, combined treatment with nicotine and IL-13 did not cause significantly higher *MUC5AC* expression than that seen after nicotine or IL-13 alone, and pretreatment with MLA blocked the increase in *MUC5AC* mRNA caused by nicotine plus IL-13 (Fig 1, D).

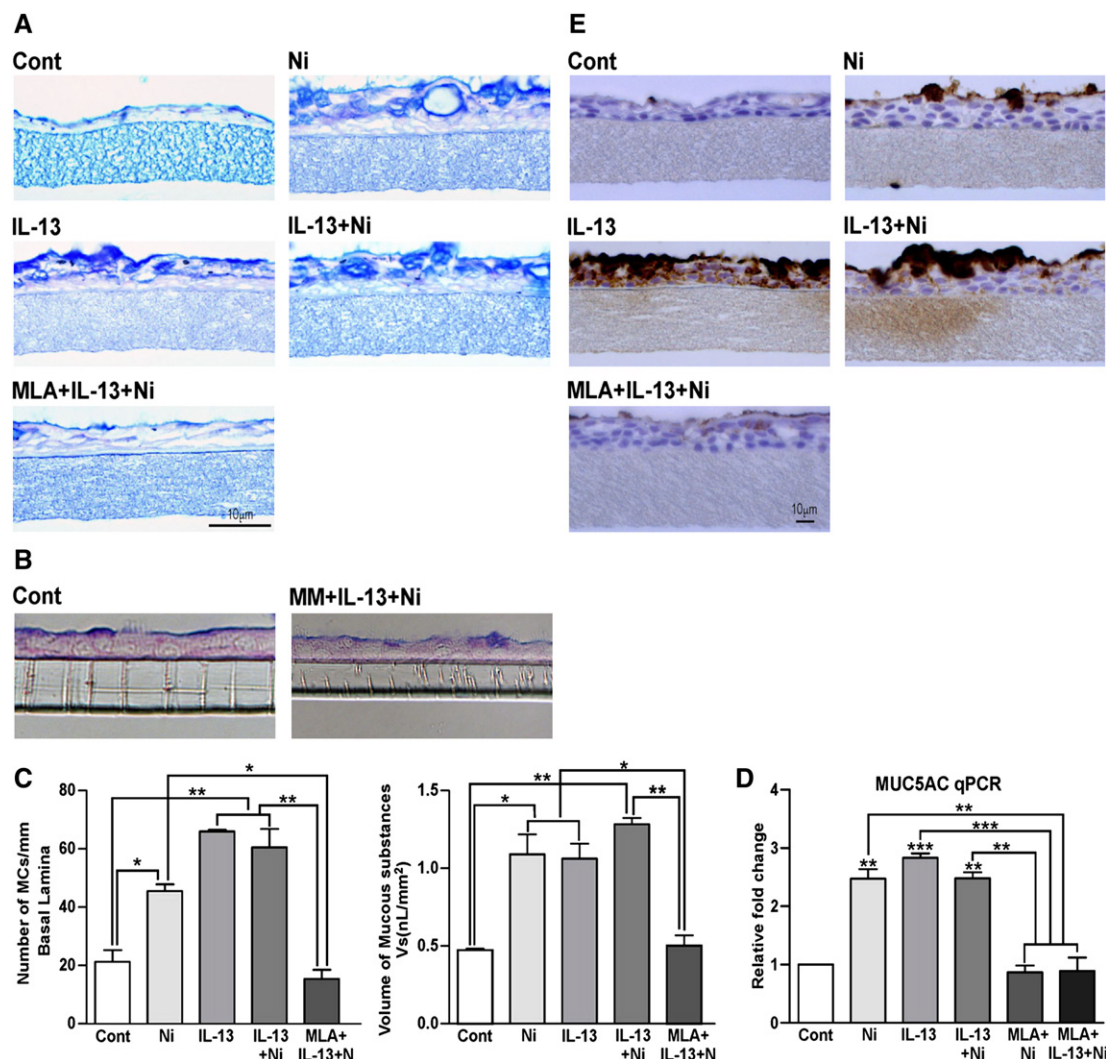


FIG 1. Nicotine (*Ni*) and IL-13 promote mucus formation in NHBE cells through nicotinic and GABA_A receptors. In NHBE cells IL-13/nicotine-induced mucus is suppressed by 1 μ mol/L MLA (**A**) and 1 μ mol/L MM (**B**). MLA blocks mucus-containing cells (**C**, left panel), mucous cell volume (Fig 1, C, right panel), MUC5AC mRNA (**D**), and MUC5AC-positive cells (**E**). Experiments were repeated at least 5 times, and bars represent means $\pm$ SEMs. * $P \leq .05$, ** $P \leq .01$, and *** $P \leq .001$.

Similar results were observed by scoring for MUC5AC protein by using IHC staining (Fig 1, E).

Nicotine-induced MUC5AC is independent of IL-13

IL-13 is the critical cytokine in mucus formation and MUC5AC expression.^{8-10,25} Although unlikely, it was possible that nicotine promoted MUC5AC/mucus formation by inducing IL-13 in NHBE cells. To ascertain this possibility, we determined IL-13 mRNA levels by using qPCR in NHBE cells before and after nicotine treatment. Unlike human Jurkat cells (positive control), qPCR analysis of NHBE cells (up to 40 cycles) did not show any detectable expression of IL-13 mRNA in the presence or absence of nicotine (see Fig E2 in this article's Online Repository at www.jacionline.org). Thus although both nicotine and IL-13 induce mucus formation and MUC5AC expression in NHBE cells, the nicotine-induced MUC5AC expression and mucus formation do not necessarily require IL-13.

$\alpha 7$ -nAChRs are required for mucus formation

Neuronal nAChRs are pentameric structures, and in mammals nAChRs are derived from 8 α -subunit ($\alpha 2$ - $\alpha 7$, $\alpha 9$, and $\alpha 10$) and 3 β -subunit ($\beta 2$ - $\beta 4$) genes; however, $\alpha 7$ and $\alpha 9$ form functional homomeric receptors.²⁶ Moreover, $\alpha 10$ subunits are functional only in the presence of the $\alpha 9$ subunit²⁷; the $\alpha 7$ and $\alpha 10$ subunits colocalize in rat sympathetic neurons.²⁸ Many nonneuronal cells, including T cells,²⁹ mast cells,³⁰ and macrophages, express nAChRs; mast cells express full-length $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ -nAChRs that respond interdependently to low concentrations of nicotine.³⁰ To ascertain whether a specific nAChR subtype mediated the effects of nicotine on mucus formation in NHBE cells, we determined the expression of nAChR subunits ($\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\alpha 9$, $\alpha 10$, and $\beta 4$) in these cells. Examining the expression of $\alpha 3$ -, $\alpha 4$ -, and $\alpha 7$ -nAChR subunits was warranted because they are expressed in the lung tracheal tissue,³¹ and genome-wide association studies show single nucleotide polymorphisms in the gene cluster encoding $\alpha 3/\alpha 5/\beta 4$ -nAChR subunits in patients

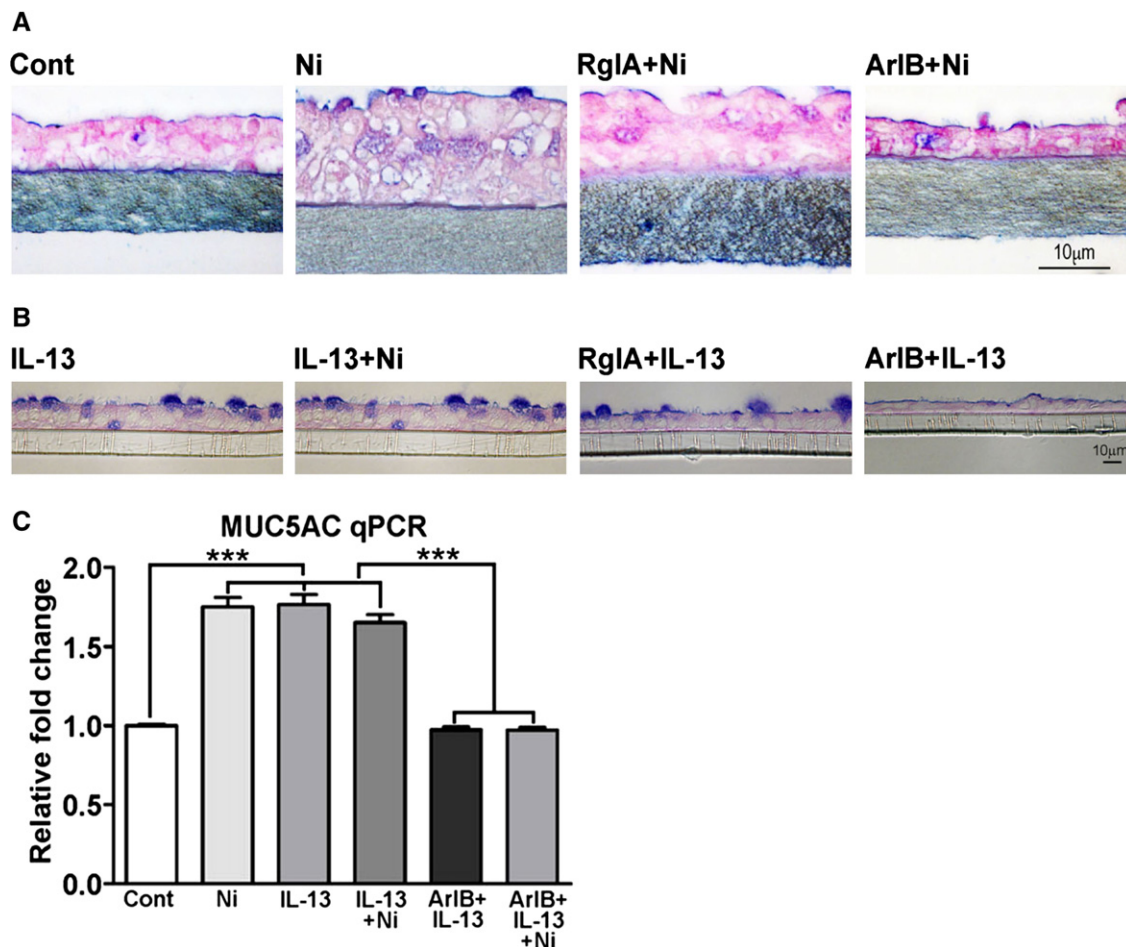


FIG 2. $\alpha 7$ -nAChR-specific conotoxin peptides suppress nicotine (Ni)/IL-13-induced mucus in NHBE cells. Effects of RgIA (500 nmol/L) and ArIB[V11L, V16D] (500 nmol/L) on nicotine-induced (A) and IL-13-induced (B) mucous cell responses and on nicotine/IL-13-induced MUC5AC mRNA by using qPCR (C) are shown. Each experiment was repeated at least 3 times, and bars on MUC5AC are means $\pm$ SEMs from 3 inserts. *** $P \leq .001$.

with lung cancer.³² Our qPCR analysis suggested that the $\alpha 3$, $\alpha 4$, $\alpha 5$, and $\beta 4$ subunits were essentially undetectable in NHBE cells (not shown); however, the cells expressed $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ -nAChR subunits (see Fig E3 in this article's Online Repository at www.jacionline.org). After normalizing with GAPDH, the mRNA expression of the $\alpha 7$ subunit was much higher than that of the $\alpha 9$ and $\alpha 10$ subunits (ie, $\alpha 7$ was detectable around 27 cycles and $\alpha 9/\alpha 10$ was detectable around 35 cycles of qPCR analysis). Thus NHBE cells express much higher levels of $\alpha 7$ than $\alpha 9/\alpha 10$ mRNA.

NHBE cells are generally refractory to various transfection/transduction approaches, and we were unable to knock down the expression of the $\alpha 7$ -, $\alpha 9$ -, or $\alpha 10$ -nAChR subunits in these cells by using siRNA (not shown). Therefore to evaluate the role of $\alpha 7$ -nAChR, $\alpha 9/\alpha 10$ -nAChR, or both in mucus production, we used receptor subtype-specific conotoxin peptides to inhibit $\alpha 7$ - and $\alpha 9/\alpha 10$ -nAChRs in NHBE cells. At lower concentrations, the conotoxin peptides ArIB[V11L, V16D] and RgIA preferentially inhibit $\alpha 7$ - and $\alpha 9/\alpha 10$ -nAChRs, respectively.^{33,34} By using these peptides at concentrations that showed minimum cross-inhibition, only ArIB[V11L, V16D] significantly reduced both nicotine-induced (Fig 2, A) and IL-13-induced (Fig 2, B) mucus production. Moreover, ArIB[V11L, V16D] (Fig 2, C), but not

RgIA (not shown), also suppressed nicotine-induced expression, IL-13-induced expression, or both of MUC5AC mRNA, as determined by using qPCR. Thus $\alpha 7$ -nAChRs are critical in the induction of airway mucus by nicotine and IL-13.

GABA_ARs are downstream of $\alpha 7$ -nAChRs

IL-13 has been shown to induce GABA_ARs in the human bronchial epithelial cell line A549.¹⁴ Although A549 cells do not produce mucus, they contain mRNAs for $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ -nAChRs (see Fig E4 in this article's Online Repository at www.jacionline.org), as well as $\alpha 3$ -, $\alpha 4$ -, and $\beta 2$ -nAChRs (not shown). Inhibiting GABA_ARs with picrotoxin (PIC) also blocked mucus production in NHBE cells.¹⁴ We used 3 approaches to determine whether the effects of nicotine on mucus formation involved GABA_ARs.

First, we showed that the nonselective GABA_AR antagonist PIC inhibited the IL-13 plus nicotine-induced mucus (AB/PAS staining; Fig 3, A) and MUC5AC proteins (IHC staining; Fig 3, B) in NHBE cells or by IL-13 and nicotine individually (see Fig E1). Similarly, PIC also blocked the IL-13 plus nicotine-induced mucous cell hyperplasia (see Fig E5, A, in this article's Online Repository at www.jacionline.org), metaplasia (see Fig E5, B), and MUC5AC mRNA expression (see Fig E5, C) in NHBE cells.

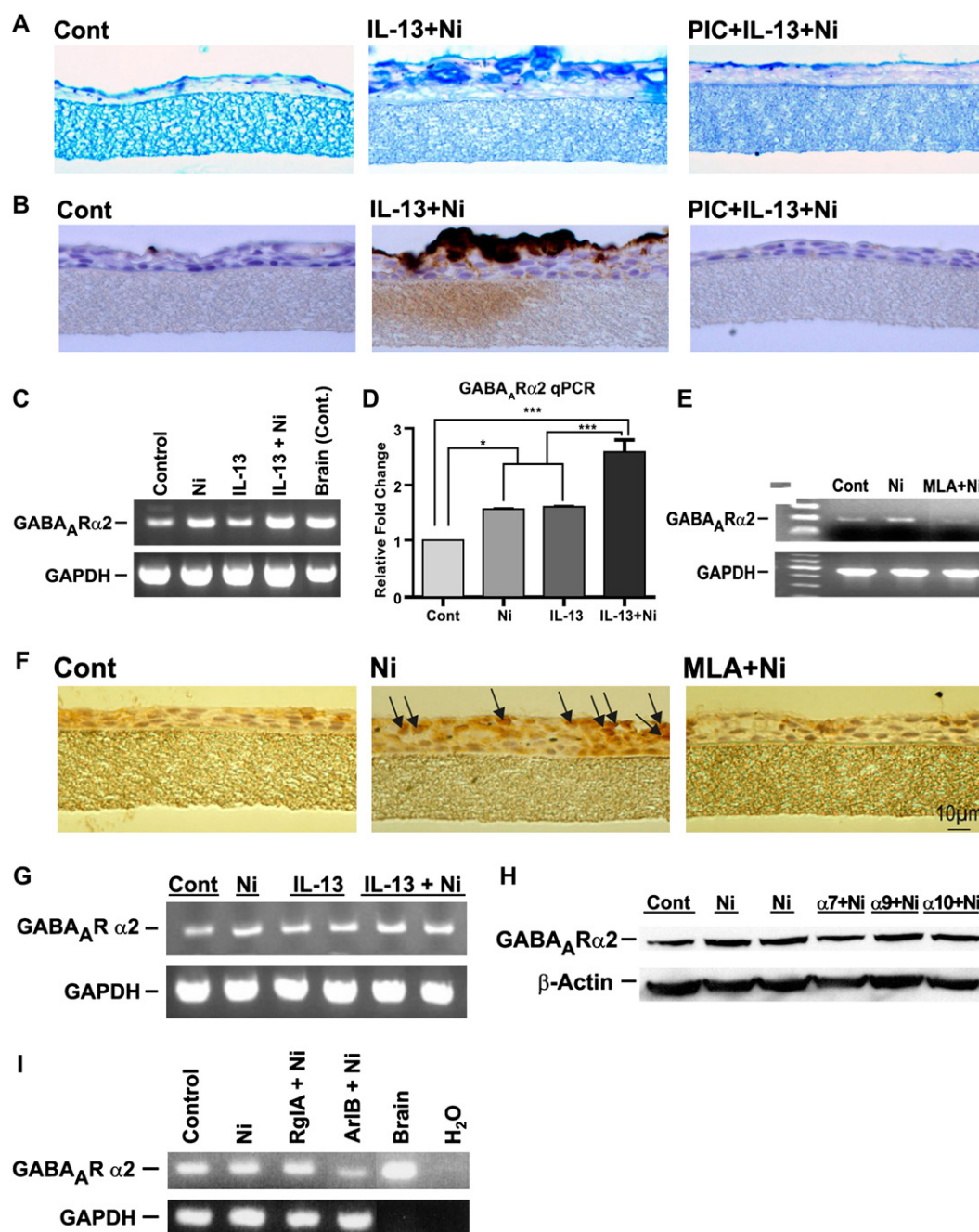


FIG 3. GABA_ARs and α7-nAChRs are critical in nicotine (Ni)/IL-13-induced mucus formation. In NHBE cells effects of 50 μmol/L PIC on mucus-positive cells (A) and MUC5AC-positive cells (B) are shown. Nicotine/IL-13-induced GABA_Aα2 expression is detected by using RT-PCR (C), qPCR (D), and IHC (E) and blocked by 1 μmol/L MLA (F). In A-549 cells nicotine/IL-13-induced GABA_Aα2 (G) is blocked by siRNA knockdown of α7-nAChRs (H) and α7-nAChR-specific conotoxin peptide ArlB[V11L, V16D] (I). **P* ≤ .05 and ****P* ≤ .001.

Second, in NHBE cells nicotine, IL-13, or both induced the expression of GABA_Aα2, as detected by using RT-PCR (Fig 3, C) and qPCR (Fig 3, D); the nicotine-induced expression of GABA_Aα2 mRNA seen by RT-PCR was blocked by the α7/α9/α10-nAChR-specific antagonist MLA (Fig 3, E). Moreover, as assayed with IHC, MLA also blocked the nicotine-induced expression of GABA_Aα2 in NHBE cells (Fig 3, F).

Third, A549 and NHBE cells express a number of GABA_AR subtypes. RT-PCR analysis suggested that both nicotine and

IL-13 upregulated the expression of GABA_Aα2 in A549 cells (Fig 3, G). Indeed, among several GABA_AR subtypes (GABA_Aα2, GABA_Aβ2, GABA_Aγ, and GABA_Aπ) only the expression of GABA_Aα2 was consistently upregulated by IL-13 and nicotine in these cells (not shown). Moreover, MLA suppressed GABA_Aα2 expression in A549 cells (see Fig E6 in this article's Online Repository at www.jacionline.org). Thus α7-nAChR, α9/α10-nAChRs, or both are involved in the increased expression of GABA_Aα2 by nicotine in A549 cells.

To identify the type of nAChR subtype among the $\alpha 7$, $\alpha 9$, and $\alpha 10$ subunits that regulates GABA_AR $\alpha 2$ expression and mucus formation, we used an siRNA approach to individually knock down $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ -nAChRs in A549 cells. Specific siRNA treatment selectively decreased the mRNA expression of $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ -nAChRs by approximately 65%, 80%, and 70%, respectively (see Fig E7 in this article's Online Repository at www.jacionline.org). As seen by using Western blotting (Fig 3, H), the knockdown of $\alpha 7$, but not $\alpha 9$ and $\alpha 10$, significantly decreased nicotine-induced expression of GABA_AR $\alpha 2$ in A549 cells. In these cells the nicotine-induced expression of GABA_AR $\alpha 2$ mRNA was also blocked by the $\alpha 7$ -specific α 7B[11L, V16D] but not the $\alpha 9/\alpha 10$ -specific RgIA conotoxin peptide (Fig 3, I). Because MLA blocked both nicotine- and IL-13-induced expression of GABA_AR $\alpha 2$, activation of nAChRs must precede the activation of GABA_AR $\alpha 2$ during mucus formation.

MM blocks GABA_AR $\alpha 2$ and mucus production in mice

Cigarette smoke promotes goblet cell hyperplasia and mucus formation.³⁵ Similarly, sensitization with allergens, such as OVA, ragweed, and *Aspergillus* species extracts, promotes mucus formation in airways.^{11,16} As seen by using immunostaining, a 2-week inhalation exposure of OVA-TCR transgenic mice on the BALB/c background to OVA, SS, or both strongly upregulated GABA_AR $\alpha 2$ expression (Fig 4, A) and mucus formation (Fig 4, B) in the airways. However, when the animals were pretreated with the nAChR antagonist MM before exposure to SS plus OVA, the amplifying effects of SS plus OVA on GABA_AR $\alpha 2$ expression (Fig 4, A) and airway mucus formation (Fig 4, B) were lost. Moreover, Western blot analysis of the lung extracts also indicated that MM blocked the OVA- and SS-induced expression of GABA_AR $\alpha 2$ (Fig 4, C). MM by itself had no detectable effect on normal bronchial epithelium, such as gross histopathology or nuclear condensation (not shown). Similarly, qPCR analysis indicated that MM also blocked the SS-induced, OVA-induced, or both Muc5ac mRNA expression in the lung (Fig 4, D). In a separate experiment lungs from BALB/c mice exposed to air (control) or nicotine inhalation (1.5 mg/m³ for 6 h/d) for 2 weeks exhibited increased expression of GABA_AR $\alpha 2$ protein by using Western blot analysis (Fig 4, E) and Muc5ac expression by using qPCR (Fig 4, F). Although an OVA-TCR transgenic murine model has been used extensively for delineating the mechanism of allergic asthma, a weakness of this model is that more than 90% of the peripheral T cells in these mice are directed to OVA and do not necessarily require sensitization with an allergen.³⁶ Therefore it is possible that the results might not be applicable to normal antigenic/allergic responses. To address this possibility, we used *A fumigatus* extracts containing the allergen in allergic bronchopulmonary aspergillosis³⁷ as the sensitizing allergen in normal BALB/c mice.¹⁶ Results presented in Fig 5 suggest that, as in the OVA transgenic system, MM suppressed the mucus formation in response to *Aspergillus* species in normal BALB/c mice, including the increase in expression of Muc5ac (Fig 5, A), airway mucus formation by AB/PAS (Fig 5, B), and GABA_AR $\alpha 2$ IHC staining (Fig 5, C). In addition, MM inhibited the inflammatory response and *Aspergillus* species-induced increase in eosinophil and lymphocyte counts, as determined by using BAL cell differential count (see Fig E8, A, in this article's Online Repository at www.jacionline.org), and the expression of the

proinflammatory cytokines IL-13 (see Fig E8, B) and IFN- γ (see Fig E8, C) in the lung. Together these results suggest that nicotine promotes GABA_AR $\alpha 2$ and mucus expression similar to that seen after exposure to cigarette smoke, and nAChRs play a critical role in both allergen- and cigarette smoke/nicotine-induced airway mucus formation *in vivo*.

Role of acetylcholine in mucus formation in NHBE cells

If the activation of nAChRs on airway epithelial cells were to play a decisive role in mucus formation, even in the absence of nicotine/cigarette smoke (eg, nonsmokers), it is important to understand how mucus-promoting molecules, such as allergens, would activate nAChRs *in vivo*. Nicotine is not normally found in mammals; rather, nicotinic cholinergic transmission is mediated by the neurotransmitter acetylcholine.³⁸ There is increasing evidence that airway epithelial cells have the enzymes to synthesize, degrade, and transport acetylcholine.^{12,39} qPCR analysis indicated that NHBE cells express primarily M3 and lower levels of M1 and M2 muscarinic receptors (see Fig E9, A, in this article's Online Repository at www.jacionline.org). Acetylcholine is a highly labile molecule and difficult to assay. Therefore to ascertain that acetylcholine promotes mucus formation in airway epithelial cells, we determined first whether inhibiting the degradation of acetylcholine through the acetylcholinesterase inhibitor NB promoted mucus formation in NHBE cells. NHBE cells were treated with indicated concentrations of NB in the absence of IL-13 or nicotine. As little as 5 μ mol/L NB (Fig 6, A) significantly upregulated the mucus formation in NHBE cells. NB also increased MUC5AC mRNA to less impressive but highly statistically significant level (Fig 6, B). Second, acetylcholine (100 μ mol/L) was added to NHBE cells, and 48 hours later, the cells were analyzed for MUC5AC and GABA_AR $\alpha 2$ mRNA expression by using qPCR. Compared with control cells, addition of acetylcholine significantly increased the expression of both MUC5AC and GABA_AR $\alpha 2$ by approximately 2-fold (see Fig E9, B and C). These results suggest that acetylcholine is a trigger for mucus formation in airway epithelial cells. However, it should be noted that we were unable to detect acetylcholine in NHBE cells in the presence of NB and IL-13. It is likely that the cellular level of acetylcholine in these cells is lower than the sensitivity of our HPLC assay.

Acetylcholine is the biological ligand for both nicotinic and muscarinic receptors. NHBE cells were treated with the nonselective muscarinic receptor antagonist atropine before NB or IL-13 to ascertain that acetylcholine mediates its promucoid effects through nAChRs. Fig 6, C, shows that atropine had no significant effect on the MUC5AC mRNA expression induced by NB, IL-13, or NB plus IL-13. Similarly, atropine did not affect mucus formation (AB/PAS staining) in NHBE cells in response to NB or IL-13 (Fig 6, D). Moreover, unlike MLA, the increased level of GABA_AR $\alpha 2$ in NHBE cells in response to nicotine or IL-13 plus nicotine was not affected by atropine treatment (Fig 6, E). These results suggest that, in the absence of nicotine or nicotine-containing substances, acetylcholine might be the critical molecule in the activation of nAChRs for mucus production in NHBE cells.

DISCUSSION

nAChRs are seen in tissues from both vertebrates and invertebrates, such as nematodes and insects.⁴⁰ In the central nervous

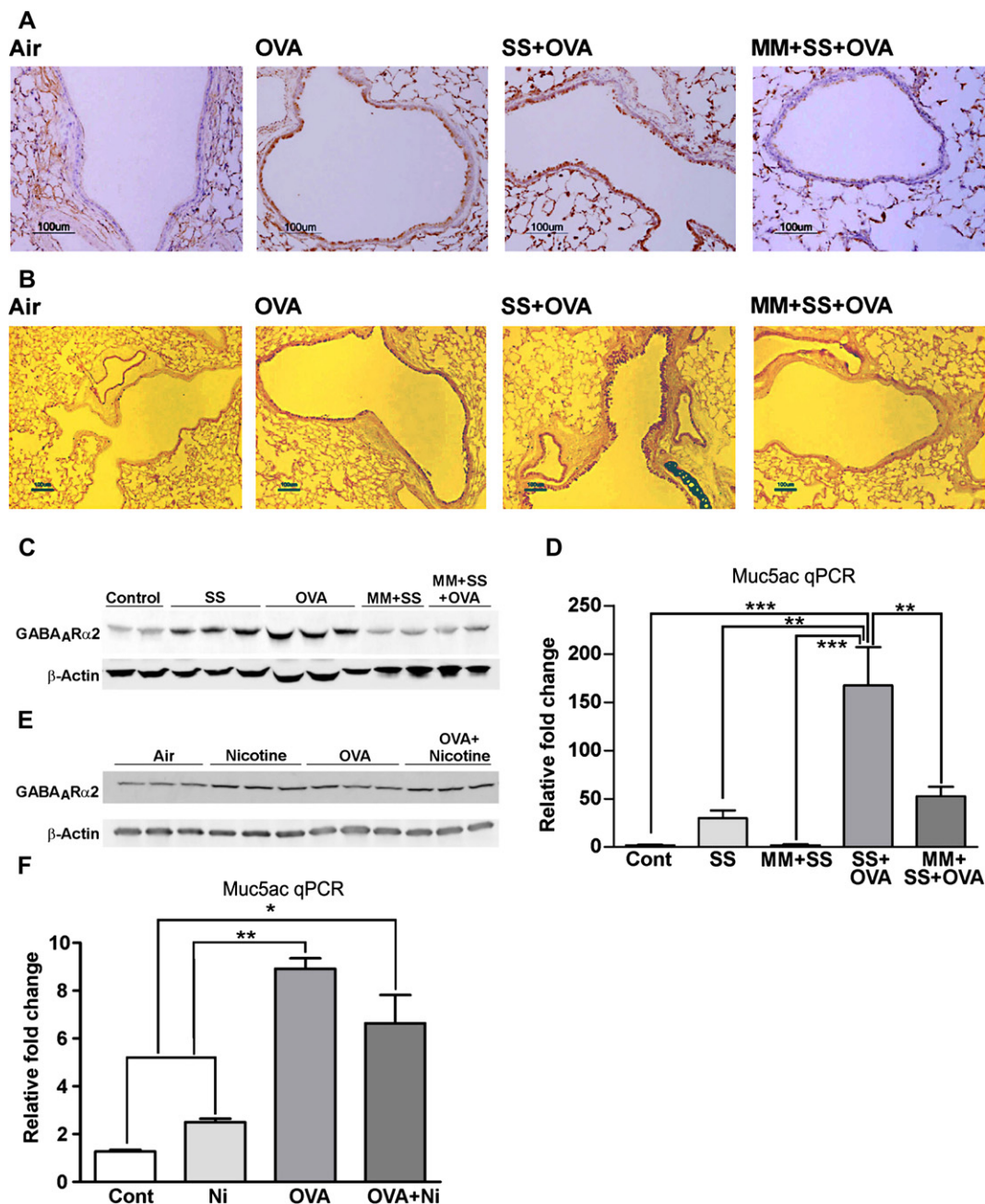
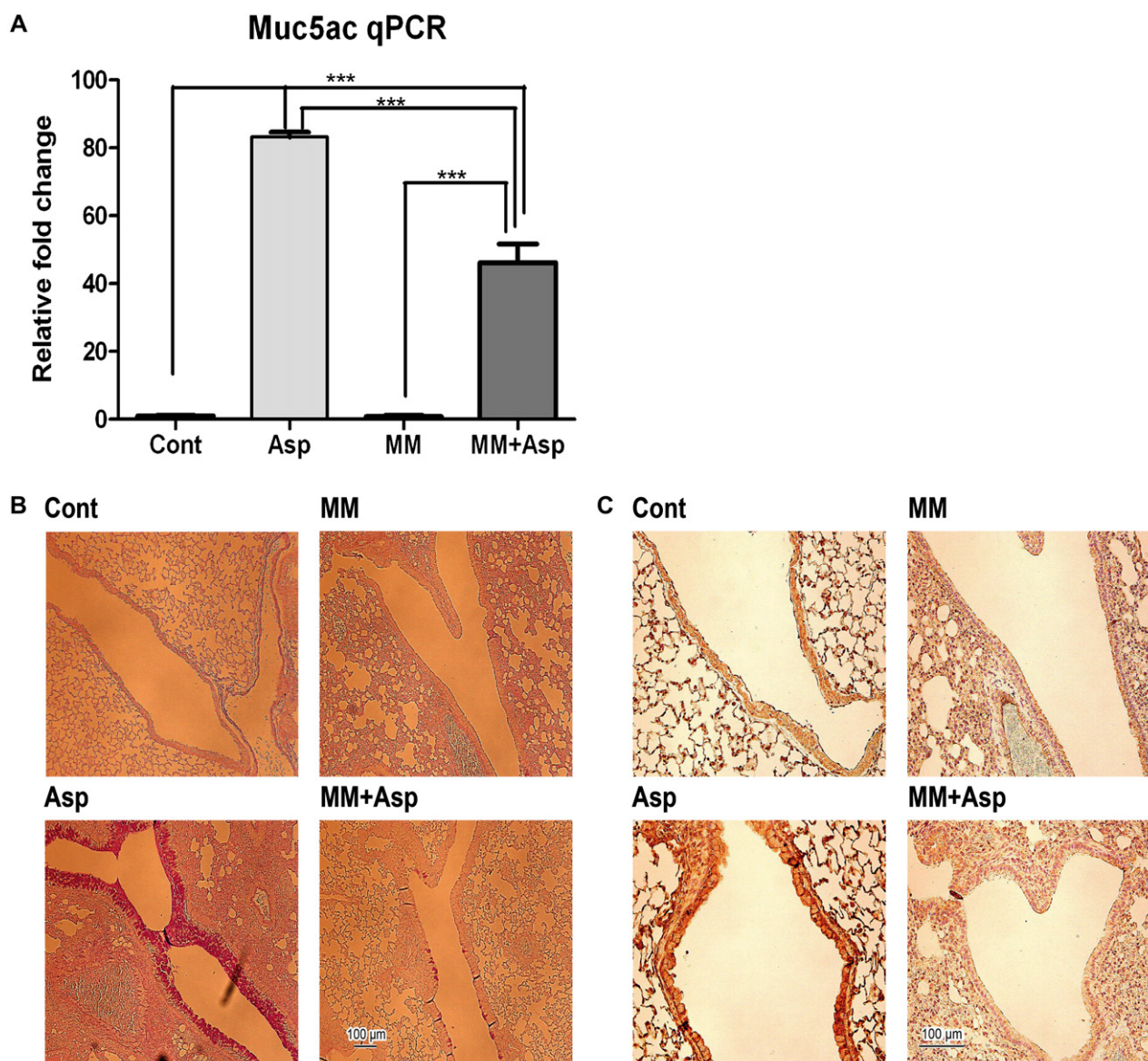


FIG 4. MM blocks OVA/SS/nicotine (Ni)-induced airway mucus, Muc5ac, and GABA_ARα2 expression in OVA-transgenic mice. Mice received indicated treatments, and lungs were tested for GABA_ARα2 by using IHC (**A**), mucus by using AB/PAS (**B**), GABA_ARα2 by using Western blots of lung extracts (**C**), levels of Muc5ac by using qPCR (**D**), GABA_ARα2 by using Western blots of lung extracts (**E**), and levels of Muc5ac by using qPCR (**F**). The results represent 2 independent experiments (3-6 mice per group). **P* ≤ .05, ***P* ≤ .01, and ****P* ≤ .001.

system nAChRs are ligand-gated ion channels that mediate fast synaptic cholinergic transmission, and these properties are strongly conserved across species. nAChRs are present in neuronal³² and many nonneuronal^{13,29,30} cell types. At least in some nonneuronal cell types nAChRs are not ligand-gated ion channels but signal through second messengers.²⁹ Thus far, the function of nAChRs in nonneuronal cells is described primarily as regulatory⁴¹; however, the near-ubiquitous presence of nAChRs in various cell types and their evolutionary conservation suggest that

these receptors might have some critical functions outside the central nervous system.⁴² Results presented herein indicate that nAChRs are essential for airway mucus production, as well as mucous cell hyperplasia and metaplasia, in response to nicotine and allergen *in vivo*, *in vitro*, or both, and it is likely that nAChRs are critical in other cellular functions.

Nicotine is immunosuppressive and anti-inflammatory,⁴¹ and our previous *in vivo* experiments indicated that chronic low-dose nicotine exposure significantly inhibited some parameters of



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FIG 5. *Aspergillus* species induces MM-sensitive GABA_Aα2 expression and mucus formation in the lung. BALB/c mice were sensitized with *Aspergillus fumigatus* (*Asp*) extracts and, where indicated, received MM. Lungs were tested for Muc5ac by using qPCR (**A**), mucus by using AB/PAS staining (**B**), and GABA_Aα2 by using IHC (**C**). The results represent 2 independent experiments with 3 to 6 mice per group. *** $P \leq .001$.

allergic asthma, including a dramatic reduction in levels of T_H2 cytokines/chemokines, such as IL-13, IL-4, IL-5, and eotaxin, and atopy, yet the nicotine-treated rats exhibited increased mucous cell metaplasia and mucus formation in the airways.¹¹ Possible explanations for these paradoxical results are that nicotine either decreased the amount of IL-13 required or substituted for IL-13 in mucus production. Our results clearly indicate that nicotine acts independently of IL-13 in promoting mucus formation and mucous cell metaplasia/hyperplasia. The ability of nAChR inhibitors to block nicotine- and IL-13-induced mucus production suggests that both IL-13 and nicotine activate nAChRs to trigger mucus formation, and IL-13's effects are upstream of nAChRs.

Previous studies have shown that IL-13 affects mucus by increasing GABA_AR expression in NHBE cells.¹⁴ We showed

that GABA_AR activation is downstream of nAChR activation in mucus formation and MUC5AC expression, and of the known GABA_AR subtypes expressed in NHBE cells, GABA_Aα2 was the only one that was significantly upregulated by IL-13 and nicotine in NHBE cells. GABA_Aα2 was also the only GABA_AR subtype the expression of which was increased by OVA, second-hand cigarette smoke, or both in OVA-TCR transgenic BALB/c mice. The interaction between nAChRs and GABA_ARs has been shown in the central nervous system,⁴³ and in *Caenorhabditis elegans* cholinergic motor neurons activate GABAergic neurons.⁴⁴ Moreover, rhesus monkeys exposed prenatally to nicotine show increased GABA signaling in the lungs⁶; however, the significance of this observation is not clear because prenatal nicotine exposure also affects the development of several organs,

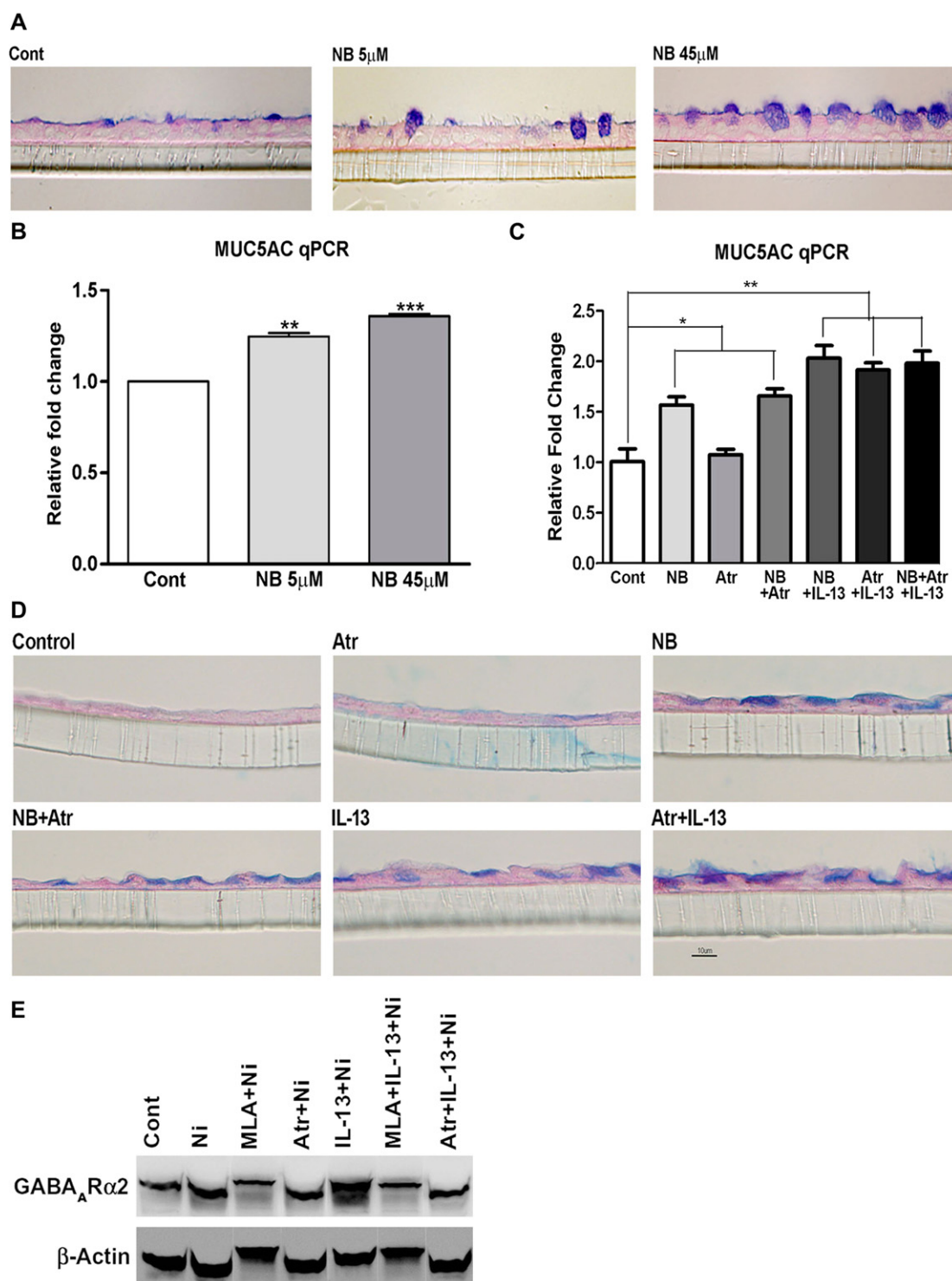


FIG 6. NB promotes mucus formation in NHBE cells. NHBE cells were treated with indicated concentrations of NB or 1 μ M/L atropine (*Atr*) and scored for NB-induced mucus (**A**) and MUC5AC (**B**). Atropine's effects on NB/IL-13-induced MUC5AC (**C**), mucus (**D**), and GABAAR α 2 protein expression (**E**) are shown. The experiment in Fig 6, A, B, and E, was repeated twice. Bars represent means $\pm$ SEMs from 3 separate inserts. *Ni*, Nicotine. * $P \leq .05$, ** $P \leq .01$, and *** $P \leq .001$.

including the lung.⁴⁵ Thus although the mechanism by which nicotine promotes the GABAergic response has not been fully delineated, it is clear that GABA $_A$ R α 2 is critical in nicotine- and IL-13-mediated mucus formation.

To ascertain the role of nAChRs in the regulation of airway mucus *in vivo*, we used 2 models of allergic asthma to trigger mucus formation. OVA-TCR transgenic BALB/c mice (a frequently used model for lung allergic responses) were exposed to OVA, SS,

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METHODS

Cell cultures

NHBE cells were obtained from rejected lung transplant donor material and provided by Greg Connor of the University of Miami. Use of these cells was approved by the Institutional Review Board of Lovelace Respiratory Research Institute. Cells were grown at the ALI by using standard procedures.^{E1} Briefly, cells were seeded into 100-mm collagen-coated plastic dishes (Corning, Inc, Corning, NY) and grown in bronchial epithelial cell growth medium (Lonza, Basel, Switzerland) supplemented with bovine pituitary extract, hydrocortisone (0.5 μ g/mL), recombinant human epidermal growth factor (0.5 ng/mL), epinephrine (0.5 μ g/mL), transferrin (10 μ g/mL), insulin (5 μ g/mL), retinoic acid (0.1 ng/mL), triiodothyronine (6.5 ng/mL), gentamicin sulfate (50 μ g/mL), and amphotericin B (50 ng/mL). Cultures were maintained at 37°C in 5% CO₂, and the medium was changed every alternate day until the cells reached approximately 90% confluence. Cells were harvested, and 2.5×10^5 cells were seeded onto a 12-mm collagen-coated Corning Costar Transwell-Clear insert with 0.4- μ m pores and submerged in the differentiation medium (1:1 Dulbecco modified Eagle medium and bronchial epithelial cell growth medium with the above supplements except gentamicin sulfate and amphotericin B and with 50 nmol/L all-trans retinoic acid and 1.5 μ g/mL BSA added). After 7 days, the medium was removed from the top side of the insert to allow cell differentiation and establish the ALI. The culture medium was replaced every alternate day, and the apical surfaces of the cells were rinsed with PBS once every week. Alternatively, predifferentiated NHBE cells (EpiAirway Tissue Model) were purchased from Mattek (Ashland, Mass). A549 cells (CCL-185) were purchased from ATCC (Manassas, Va) and grown according to the vendor's directions.

Animal studies

OVA-TCR transgenic hemizygous mice (DO11.10) on a BALB/c background were bred in our animal facility by mating DO11.10 +/- hemizygous males with BALB/c females. Mice were exposed in whole-body inhalation exposure chambers (Hazelton H2000, Lab Products, Maywood, NJ) to air, SS, or nicotine (1.5 mg total particulate material/m³), heat-aggregated OVA aerosol (5 mg/m³), or OVA plus SS or nicotine for 2 weeks (6 h/d for 5 d/wk). In some experiments normal (wild-type) BALB/c mice were sensitized to *A fumigatus* (Greer Laboratories, Lenoir, NC) extract, as described previously.^{E2} Briefly, the allergen used in the study was a lyophilized culture filtrate preparation of *A fumigatus*; the filtrates were stored at -70°C until use. Mice were immunized intratracheally with *A fumigatus* extract (50 mg/0.1 mL of endotoxin-free sterile saline) or sterile saline alone and subsequently challenged with the *A fumigatus* extract (100 mg/0.1 mL administered intratracheally) 3 times at 5-day intervals. For MM treatment, mice were subcutaneously implanted with MM-containing miniosmotic pumps 3 weeks before exposures, as previously described.^{E3} The pumps delivered 2 mg of MM/kg body weight per day. The trachea was surgically exposed and cannulated and the right lobe was lavaged twice with 1 mL of sterile Ca²⁺/Mg²⁺-free PBS (pH 7.4) to collect lung lavage fluid. Total and differential BAL cells were counted microscopically.^{E2} The left lung was fixed and the right lung was processed for RNA and protein extraction.^{E4} All animal protocols used in this study were approved by the Institutional Animal Care and Use Committee of the Lovelace Respiratory Research Institute.

Mucus staining and IHC

NHBE cells at the ALI were fixed in 10% neutral buffered formalin and embedded in paraffin; 5- μ m-thick sections were stained for mucus with AB/PAS.^{E5,E6} Briefly, after deparaffinization, the slides were stained with AB solution at pH 2.5 for 30 minutes, and after washing, they were treated with 1% periodic acid for 10 minutes. The slides were stained with Schiff reagent for 10 minutes, rinsed 3 times with sodium metabisulfite, washed, dehydrated, mounted, and examined microscopically at the indicated magnification. To quantify mucus-producing cells and mucus volume, we determined the number of AB/PAS-positive cells per millimeter of basal lamina and the mucus volume per cubic meter of basement membrane. The tissue sections were examined and quantified blind by using an Olympus BH-2 light microscope (Olympus, Center Valley, Pa) equipped with the National Institutes of Health image analysis system, as described previously.^{E6}

Immunohistochemical staining was performed according to the protocol previously described.^{E7} Mouse monoclonal anti-MUC5AC (45M1; Thermo Scientific, Lafayette, Colo) and rabbit polyclonal anti-GABA_AR α 2 (Sigma, St Louis, Mo) antibodies were used at preoptimized concentrations of 1:100 and 1:50 dilution, respectively. The tissue sections were treated with the primary antibodies overnight at 4°C, followed by incubation with biotinylated secondary antibody (VECTASTAIN Elite ABC kit; Vector Laboratories, Burlingame, Calif). Binding was visualized with an avidin-biotinylated enzyme complex (VECTASTAIN Elite ABC kit), with 3, 3'-diaminobenzidine as substrate.

Cell treatments

Cells were treated with 100 nmol/L nicotine base (Sigma), 10 to 50 ng/mL recombinant human IL-13 (R&D Systems, Minneapolis, Minn), or indicated concentrations of NB, respectively, and the cultures were harvested 48 hours later. GABA_AR, nAChR, or muscarinic receptor inhibitors were added at the indicated concentrations 2 hours before the addition of IL-13, nicotine, or NB.

RT-PCR and qPCR

Total RNA from murine lungs, NHBE cells, and A549 cells was isolated by using TRI-Reagent. One-step RT-PCR was performed with the SuperScript III One-Step RT-PCR System with Platinum Taq. RT-PCR primers for GABA_AR α 2 were 5'-AGGCTTCCGTATGATACAG (forward) and 5'-AGGACTGACCCCTAATACAG (reverse), and those for GAPDH were 5'-CCCATCACCACCTCTTCCAGGAG (forward) and 5'-TTCACCACCTTCTTCTGATGTCAT (reverse); primers were purchased from Sigma.

qPCR was performed with the Step One plus Detection System (Applied Biosystems, Foster City, Calif) and the TaqMan One-Step RT-PCR kit containing AmpliTaq Gold DNA polymerase. Specific primers and probes for MUC5AC; the nAChR α 7/ α 9/ α 10 subunits; muscarinic receptors M1, M2, M3; and GABA_AR α 2 (forward 5'-CCACCATCTCCAAGAGTGCAA and reverse 5'-TGCTTCAGCTGGCTTGTTTC and probe CAGCCGAGAACC-CAACAAGAAGCC) for qPCR were obtained from Applied Biosystems. Fold differences were determined by using the $2^{(-\Delta\Delta CT)}$ method.^{E8}

Knockdown of α 7-, α 9-, and α 10-nAChRs with siRNAs

siRNAs were purchased from Thermo Scientific. The siRNA SMART-pool contained 4 pooled siRNA duplexes with "UU" overhangs and a 5' phosphate on the antisense strand. Briefly, the siRNA-DharmaFECT 1 complex was added to the cells in complete medium and incubated at 37°C in 5% CO₂ for 48 hours. The mRNA levels of α 7-, α 9-, and α 10-nAChRs were assessed 48 hours after transfection with TaqMan One-Step RT-PCR. Protein levels of GABA_AR α 2 were determined by using Western blot analysis.

Western blot analysis

Western blot analysis was performed as described previously.^{E7} Briefly, lung tissues were homogenized in RIPA buffer (20 mmol/L Tris, 150 mmol/L NaCl, 20 mmol/L β -glycerol-phosphate, 1% Triton-X, 10 mmol/L NaF, 5 mmol/L EDTA, and 1 mmol/L Na₃VO₄) containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride and 1 μ g/mL each of aprotinin, antipain, and leupeptin) at 4°C. The protein content of the extracts was determined by using the BCA Protein Assay Kit (Pierce, Rockford, Ill). Samples were electrophoresed on 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, Calif). Nonspecific binding was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) for 30 minutes at room temperature, followed by overnight incubation with the indicated specific primary antibody at 4°C. GABA_AR α 2 expression was determined by probing the blot with anti-GABA_AR α 2 murine mAb (Millipore, Temecula, Calif). The blots were washed with TBST (3 $\times$), incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibody, and washed with TBST (3 $\times$). Blots were developed

with ECL (GE Healthcare, Hertfordshire, United Kingdom) with x-ray photo film.

Statistical analysis

All data were analyzed with GraphPad Prism software 5.03. One-way ANOVA was used to compare means between the groups by using the Tukey *post hoc* test, which compares all groups at 95% CIs. Results are presented as means $\pm$ SDs. Differences with a *P* value of less than .05 were considered statistically significant.

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- E8. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402-8.

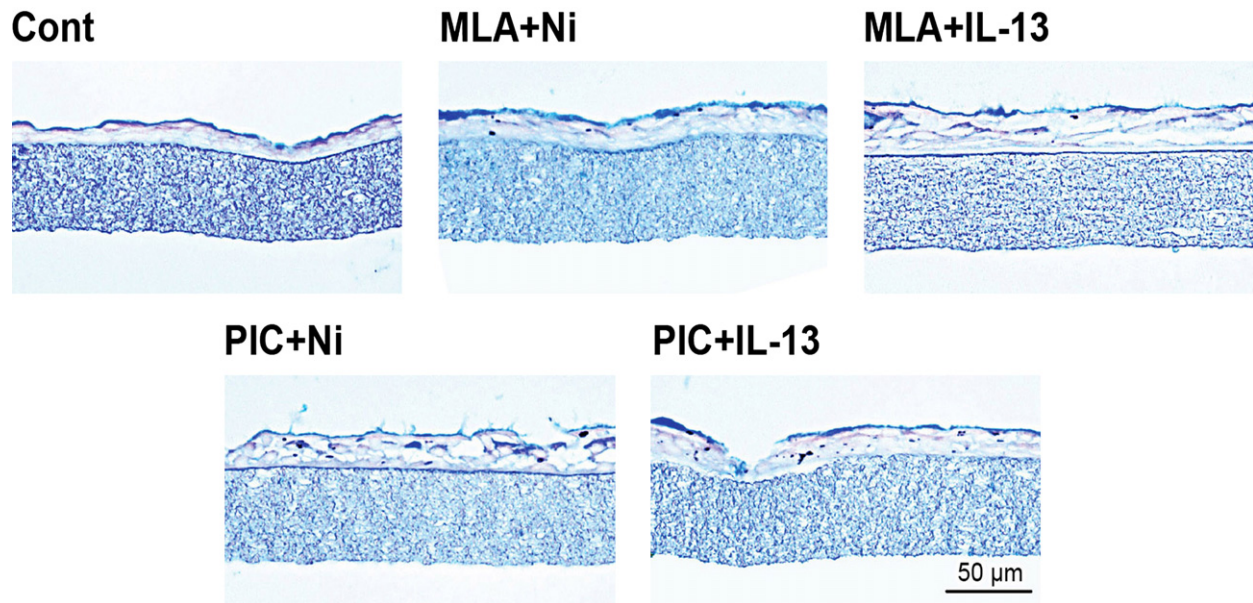


FIG E1. MLA and PIC block the nicotine (*Ni*)– and IL-13–induced mucus formation in NHBE cells. NHBE cells were cultured at the ALI with 100 nmol/L nicotine and 50 ng/mL IL-13 for 48 hours. Where indicated, 1 $\mu\text{mol/L}$ MLA or 50 $\mu\text{mol/L}$ PIC was added to cultures 2 hours before nicotine, IL-13, or both. The *inserts* were fixed and stained for mucus (AB/PAS).

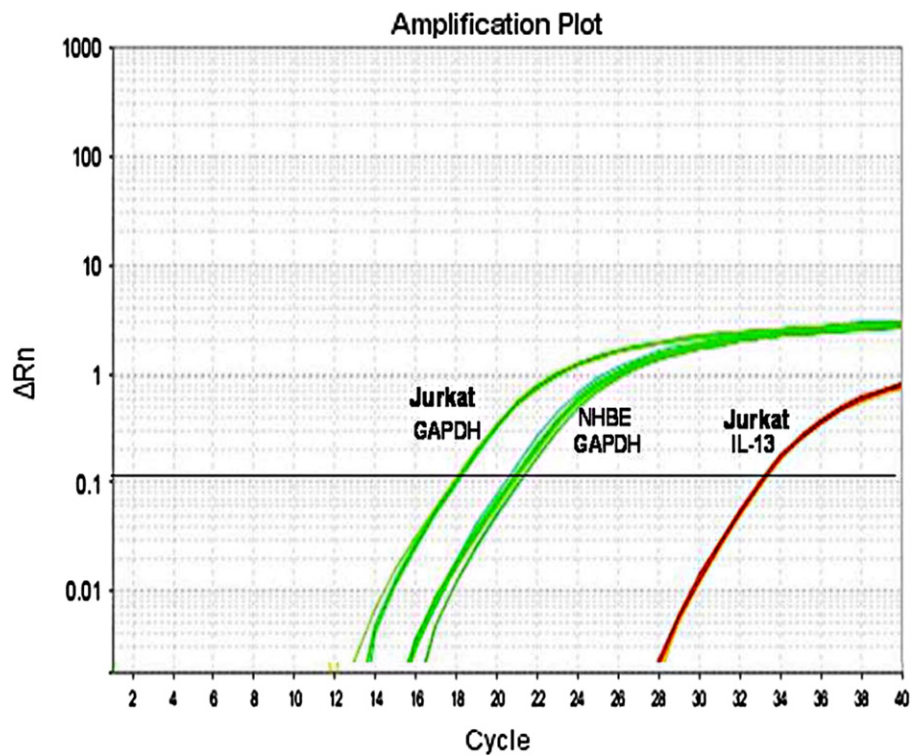


FIG E2. Unlike human Jurkat T cells, NHBE cells do not express the IL-13 gene, even after nicotine treatment. RNA was isolated from Jurkat cells and NHBE cells (with and without 100 nmol/L nicotine treatment for 48 hours) and analyzed for the expression of IL-13 by using qPCR. The experiments were repeated 3 times with different batches of RNA. ΔRn , The magnitude of the signal generated by the given set of PCR conditions.

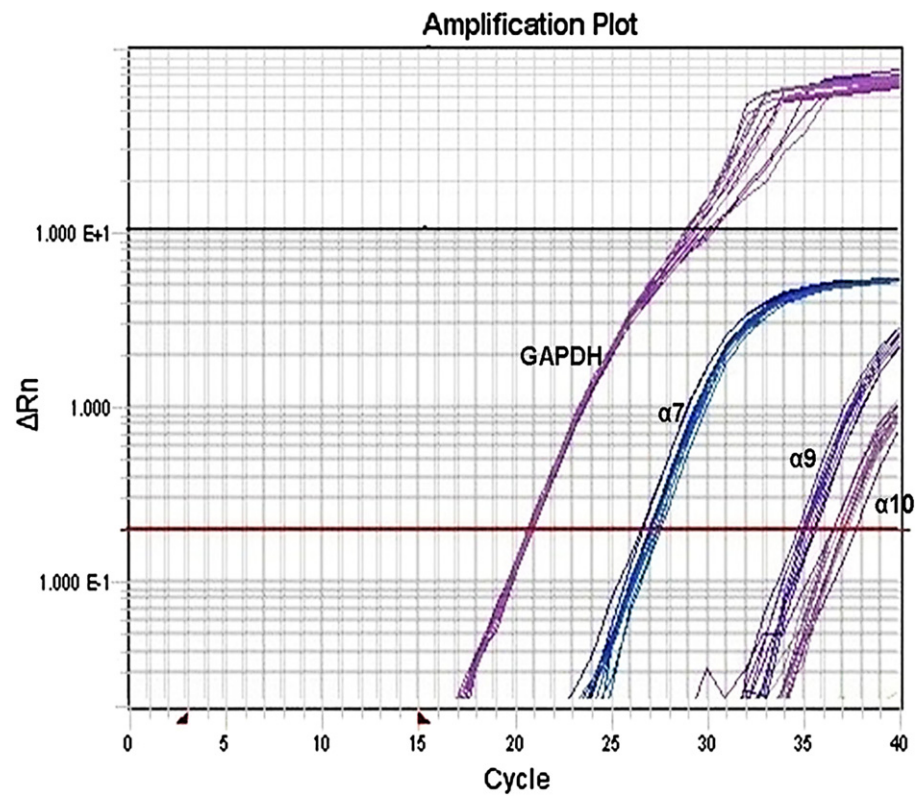


FIG E3. NHBE cells express $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ -nAChR subunits. RNA from NHBE cells was analyzed for the expression of $\alpha 3$ -, $\alpha 4$ -, $\alpha 5$ -, $\alpha 7$ -, $\alpha 9$ -, $\alpha 10$ -, and $\beta 2$ -nAChR subunits by using qPCR. Only the $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ subunits were detected within 40 cycles of amplification. ΔRn , The magnitude of the signal generated by the given set of PCR conditions.

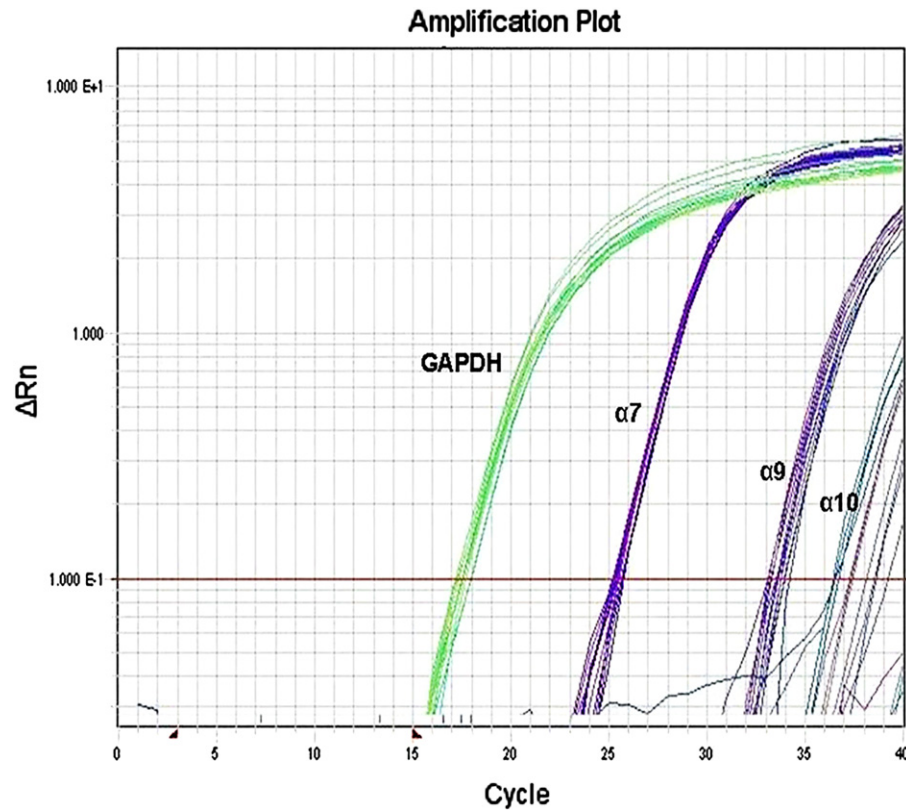


FIG E4. A549 cells express $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ -nAChR subunits. RNA from A549 contained mRNA for $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ -nAChR subunits, as detected by using qPCR. However, these cells also contained detectable levels of $\alpha 3$, $\alpha 4$, and $\beta 2$ subunit mRNA (not shown). ΔRn , The magnitude of the signal generated by the given set of PCR conditions.

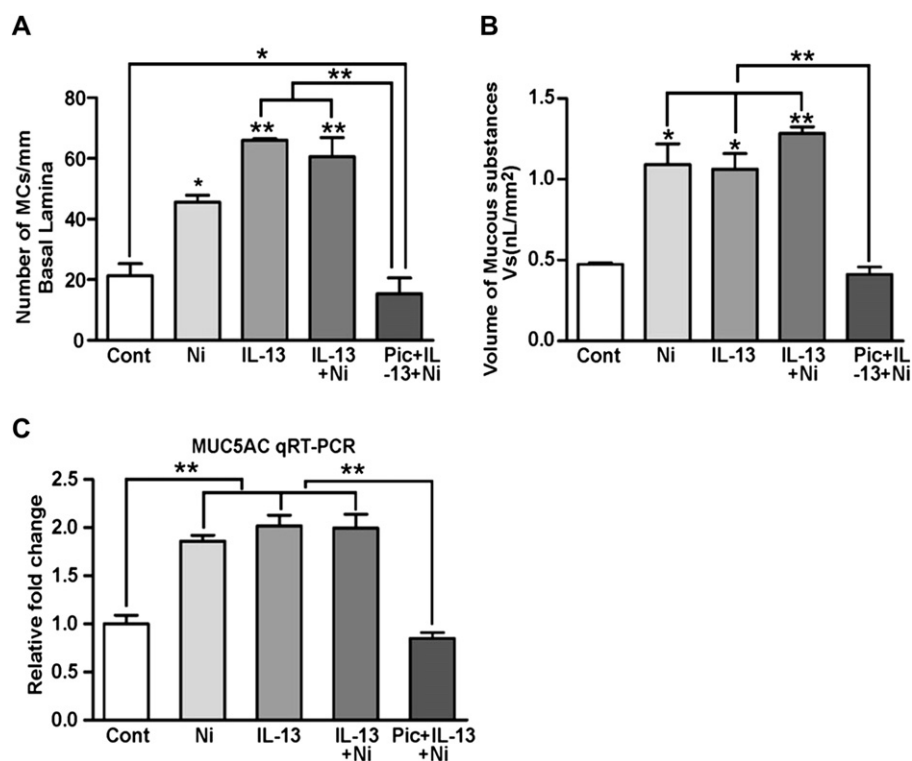


FIG E5. Picotoxin blocks the effects of nicotine (*Ni*) and IL-13 on mucus formation. NHBE cells were treated with nicotine, IL-13, or both, as described in the Methods section. In some cultures 50 μ mol/L PIC was added 2 hours before IL-13 and nicotine. The number of mucus-containing cells per millimeter of basal lamina (**A**), the volume of mucus-positive cells (**B**), and MUC5AC expression determined by means of qPCR (**C**) in the presence and absence of PIC are shown. Bars represent values $\pm$ SEMs for 3 inserts. Each experiment was repeated at least 5 times. * $P \leq .05$ and ** $P \leq .01$.

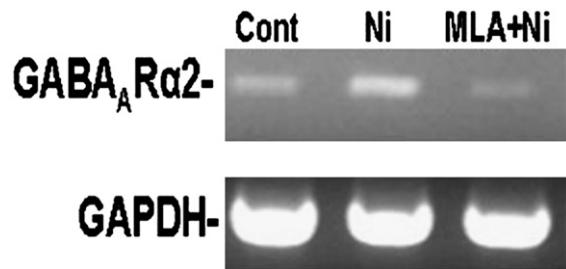


FIG E6. MLA blocks nicotine (*Ni*)-induced GABA_AR2α in A549 cells. mRNA from A549 cells after treatment with nicotine and nicotine plus MLA (1 μmol/L) was analyzed by using RT-PCR, as described in the Methods section.

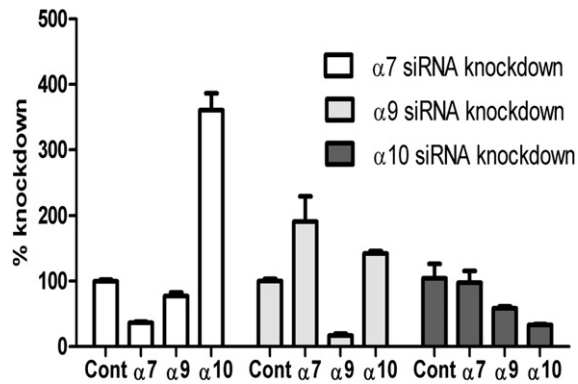


FIG E7. Specific siRNAs decrease the mRNA expression of the selected nAChR subtypes. A549 cells were treated with $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ -specific siRNA, as described in the Methods section. At 48 hours after siRNA treatment, the expression of the $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ -nAChR subunits was assayed by using qPCR. The increased expression of the $\alpha 10$ subunit after $\alpha 7$ siRNA treatment was consistently observed. The experiment was repeated twice. Bars represent means $\pm$ SEMs from 2 separate siRNA-treated cell cultures.

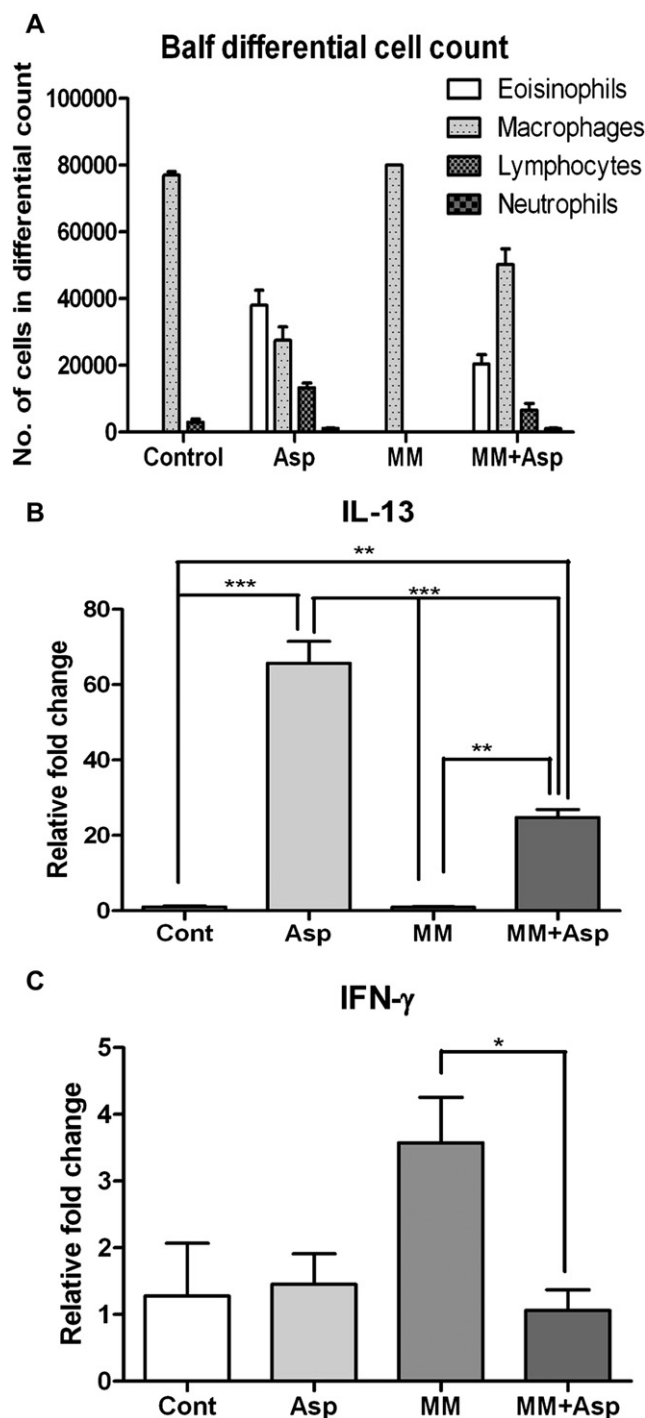


FIG E8. *Aspergillus* species (*Asp*) induce MM-sensitive GABA_A α 2 and mucus formation in the airways. BALB/c wild-type mice were exposed to *Aspergillus fumigatus* extract, as described in the Methods section. Some animals received the nAChR inhibitor MM. Differential cell counts in bronchoalveolar lavage fluid (**A**), lung IL-13 expression determined by using qPCR (**B**), and lung IFN- γ expression determined by using qPCR in various groups (**C**) are shown. The results represent 2 independent *in vivo* exposure experiments with 3 to 6 mice per group. * $P \leq .05$, ** $P \leq .01$, and *** $P \leq .001$.

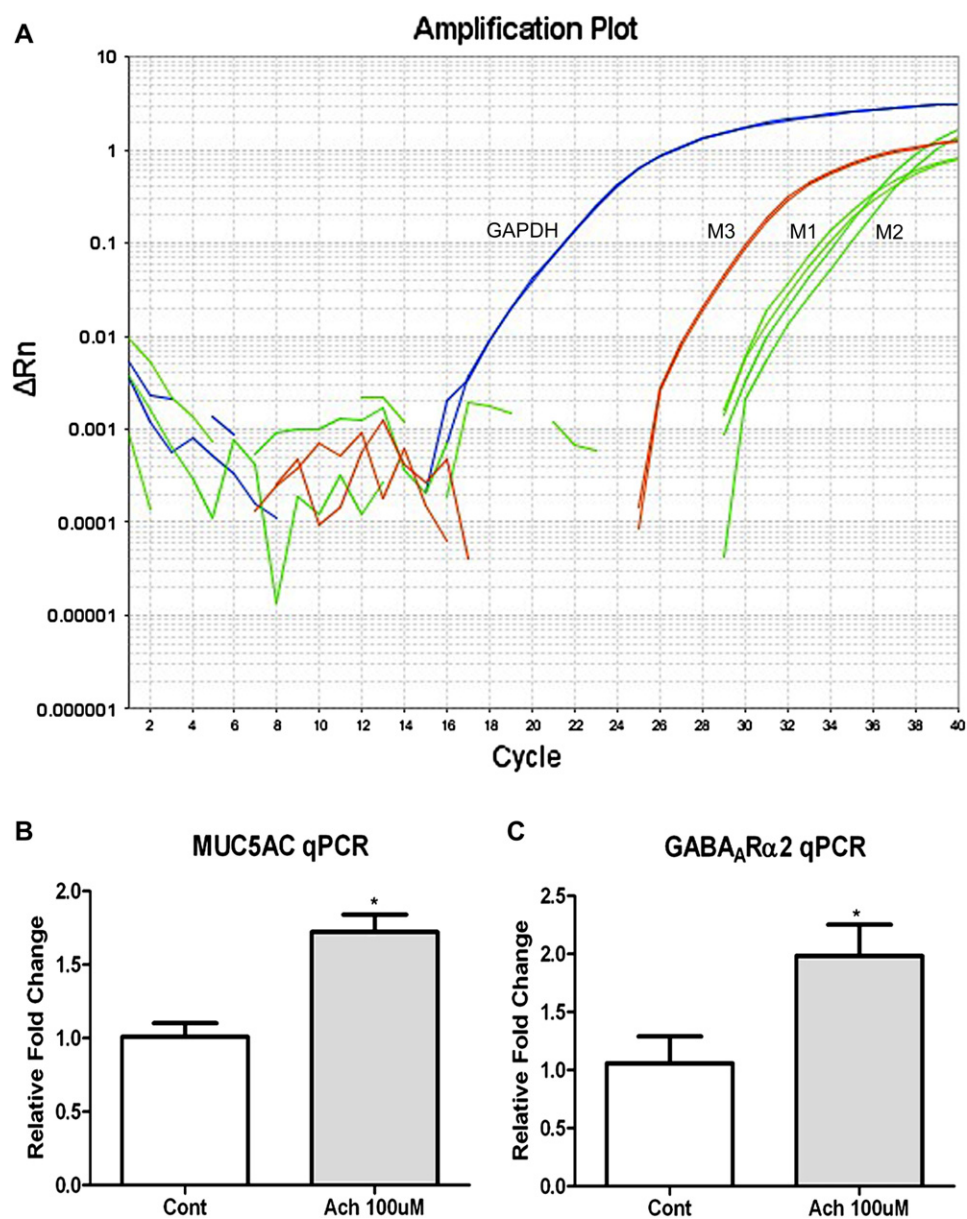


FIG E9. NHBE cells express M1, M2 and M3 muscarinic receptor subunits. **A**, RNA from NHBE cells was analyzed for the expression of M1, M2, and M3 muscarinic subunits by using qPCR. **B**, Acetylcholine (ACh; 100 μ mol/L) was added to NHBE cells, and 48 hours later, the cells were analyzed for MUC5AC and GABA_Aα2 mRNA expression by using qPCR. ΔRn , The magnitude of the signal generated by the given set of PCR conditions. * $P \leq .05$.