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Thirdhand smoke component can exacerbate a mouse asthma model through mast cells

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Background: Thirdhand smoke (THS) represents the accumulation of secondhand smoke on indoor surfaces and in dust, which, over time, can become more toxic than secondhand smoke. Although it is well known that children of smokers are at increased risk for asthma or asthma exacerbation if the disease is already present, how exposure to THS can influence the development or exacerbation of asthma remains unknown.
Objective: We investigated whether epicutaneous exposure to an important component of THS, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), can influence asthma pathology in a mouse model elicited by means of repeated intranasal challenge with cockroach antigen (CRA).
Methods: Wild-type mice, α7 nicotinic acetylcholine receptor (nAChR)– or mast cell (MC)–deficient mice, and mice with MCs that lacked α7 nAChRs or were the host's sole source of α7 nAChRs were subjected to epicutaneous NNK exposure, intranasal CRA challenge, or both, and the severity of features of asthma pathology, including airway hyperreactivity, airway inflammation, and airway remodeling, was assessed.
Results: We found that α7 nAChRs were required to observe adverse effects of epicutaneous NNK exposure on multiple features of CRA-induced asthma pathology. Moreover, MC expression of α7 nAChRs contributed significantly to the ability of epicutaneous NNK exposure to exacerbate airway hyperreactivity to methacholine, airway inflammation, and airway remodeling in this model.
Conclusion: Our results show that skin exposure to NNK, a component of THS, can exacerbate multiple features of a CRA-induced model of asthma in mice and define MCs as key contributors to these adverse effects of NNK. (J Allergy Clin Immunol 2018;■■■:■■■-■■■.)

Key words: *Thirdhand smoke, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, mast cells, α7 nicotinic acetylcholine receptor, cockroach allergen*

Exposure to environmental tobacco smoke is a significant health concern, and recent attention has focused on possible roles of thirdhand smoke (THS) in patients with tobacco-related diseases. THS is the residual tobacco smoke contamination remaining after the source of tobacco smoke has been extinguished.[1] Tobacco smoke contaminants, including nicotine, persist in places where people smoke (eg, homes and cars) and can potentially cause adverse health effects.[2-5] Nicotine adsorbed onto indoor surfaces can react with ozone and gaseous nitrous acid (a pollutant in vehicle exhaust that also is produced by improperly vented gas stoves and burning tobacco) to yield potentially bioactive residues, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 1-(N-methyl-N-nitrosamino)-1-(3-pyridinyl)-4-butanal (NNA), and N′-nitrosonornicotine, the 3 main tobacco-specific nitrosamines formed in the reaction of gaseous nitrous acid and sorbed nicotine.[6]

In the United States approximately 43% of children aged 2 months to 11 years live with a smoker.[2] Infants and young children are thought to be at greater risk for THS exposure than adults because they typically spend more time indoors, and crawling babies can absorb chemicals through the skin.[7,8] Moreover, THS exposure can cause alterations in multiple organ systems.[9] For example, exposure to THS can result in increased collagen production and levels of inflammatory cytokines in the lung.[9] THS exposure can also increase the risk of thrombosis[10] and insulin resistance[11] and impair wound healing.[12]

It is thought that the biological effects of major THS components (nicotine and its metabolites: NNK, NNA, and N′-nitrosonornicotine) are mediated by nicotinic acetylcholine receptors (nAChRs), including the high-affinity receptor for NNK, α7 nAChR.[13] In addition to neurons, many other cell types express α7 nAChR, including immune cells, such as B[14,15] and T[16,17] lymphocytes, regulatory T cells,[18] dendritic cells,[19,20] macrophages,[21] eosinophils,[22] and mast cells (MCs).[23-26] However, little is known about the mechanisms that link THS exposure to disease.

Asthma is characterized by inflammation of the airways, airway hyperreactivity (AHR), airflow obstruction, and airway wall remodeling.[27,28] MCs are cells of hematopoietic origin[29,30] that complete their maturation and then reside in virtually all tissues.[29-31] Repetitive activation of airway MCs by antigen-specific IgE and its cognate antigen is thought to play a key role in asthma pathology in many subjects,[32] and activation of MCs by IgE and specific antigen is regarded as the main process by which MCs contribute to atopic asthma and other allergic disorders.[33-38] Although mouse,[24,39,40] rat,[25,41] and human[23,42] MCs express α7 nAChRs, there have been few studies of the effects of α7 nAChR agonists on MCs, and all such data (except for a study

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Abbreviations used
AHR: Airway hyperreactivity
BAL: Bronchoalveolar lavage
BALF: Bronchoalveolar lavage fluid
BMCMC: Bone marrow–derived cultured mast cell
BW: Body weight
CRA: Cockroach antigen
MC: Mast cell
nAChR: Nicotinic acetylcholine receptor
NNA: 1-(N-methyl-N-nitrosamino)-1-(3-pyridinyl)-4-butanal
NNAL: 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNK: 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone
OVA: Ovalbumin
R_L: Lung resistance
THS: Thirdhand smoke
TLR: Toll-like receptor
WT: Wild-type

of mucosal MCs in a model of food allergy[39] and ours, as discussed below) are from *in vitro* work. Such findings indicate that MC responses to α7 nAChR signaling (eg, degranulation or cytokine production) can vary, depending on the concentration or type of α7 nAChR agonist, the type of MCs studied, and the presence or absence of concomitant IgE/FcεRI signaling.[24-26,39] Although the possible role of MCs in asthma exacerbation by THS components has not been studied, measurement of markers of asthma severity, such as exhaled nitric oxide, indicate that exposure to even low levels of THS can cause significant airway inflammation.[43]

In the present study we exposed mice repeatedly to intranasal cockroach antigen (CRA), the most important allergen for eliciting asthma in inner-city households in the United States,[44-46] to establish a mouse model of CRA-induced asthma. We then used this mouse model to define the extent to which epidermal exposure to NNK could influence the severity of asthma pathology and investigate to what extent MCs contributed to any observed effects of NNK.

METHODS

Mice

All animal experiments were carried out according to protocols approved by the Stanford University Administrative Panel on Laboratory Animal Care. Genetically MC-deficient C57BL/6 (B6)-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were generously provided by Peter Besmer (Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY); these mice were then backcrossed to C57BL/6J (B6J) mice for more than 10 generations.[47] MC-deficient B6-*Cpa3-Cre; Mcl-1*$^{fl/fl}$ mice and the MC-sufficient control strain B6-*Cpa3-Cre; Mcl-1*$^{+/+}$ were generated in our laboratory.[48] Because B6-*Cpa3-Cre; Mcl-1*$^{fl/fl}$ mice have deficiencies in MCs (and basophils) that are independent of mutations affecting Kit, we call them informally in our laboratory "Hello *Kit*ty" (H*K*) mice. FcεRIγ-deficient *Fcer1g*$^{-/-}$ mice on the C57BL/6 background (B6.129P2-Fcεr1gtm1Rav N12) were purchased from Taconic (Rensselaer, NY). The α7 nAChR–deficient B6J-*Chrna7*$^{-/-}$ mice and Toll-like receptor 4 (TLR)−deficient B6J-*Tlr4*$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, Me). B6J-*Chrna7*$^{-/-}$-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were generated by crossing B6J-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice to B6J-*Chrna7*$^{-/-}$ mice. Age-matched female mice were used for all experiments. All animal care and experimentation reported was conducted in compliance with the guidelines of the National Institutes of Health and with specific approval of the Institutional Animal Care and Use Committee of Stanford University.

For details of other methods used in this study, see the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

Both NNK and the α7 nAChR agonist PNU282987 can exacerbate allergic airway inflammation elicited after intranasal CRA challenge

In wild-type (WT) C57BL/6J (B6J) mice, our CRA-induced model (intranasal CRA challenge with 10 μg of CRA/0.03 mL of PBS on days 1, 2, 15, 18, and 21) resulted in significantly increased airway reactivity (ie, AHR) to methacholine (measured as lung resistance [R_L]) and increased numbers of total leukocytes in bronchoalveolar lavage fluid (BALF), as well as significant increases (vs mice challenged with PBS instead of CRA) in BALF neutrophils, eosinophils, and lymphocytes (Fig 1). We found that intraperitoneal application of either the α7 nAChR agonist PNU282987 (10 mg/kg body weight [BW] in 0.2 mL of normal saline 5 minutes before performing intranasal challenge with CRA on days 1, 2, 15, 18, and 21), epicutaneous painting of the left ear pinna (indicated with a red circle in Fig E1, *A*, in this article's Online Repository at www.jacionline.org) with NNK (0.5 mg/kg BW in 0.01 mL of ethanol 5 minutes before the CRA challenge on days 1, 2, 15, 18, and 21), or intraperitoneal PNU282987 injection plus epicutaneous NNK painting significantly exacerbated CRA-induced AHR (Fig 1, *A*) and increased numbers of BALF total leukocytes and neutrophils (Fig 1, *B*) versus corresponding values in mice that received neither PNU282987 nor NNK. Intraperitoneal injection of PNU282987 plus epicutaneous NNK painting also increased numbers of BALF eosinophils and lymphocytes (Fig 1, *B*). These findings indicate that exposure to NNK, the α7 nAChR agonist PNU282987, or both can exacerbate features of allergic airway inflammation, including AHR to methacholine and numbers of BALF leukocytes, in this CRA-induced mouse model of asthma.

In this model intranasal CRA challenge elicited a combination of T_H1 and T_H2 cell immune responses, as assessed in both systemic immunity and the inflamed site (the lung, see Fig E2 in this article's Online Repository at www.jacionline.org), which could be further promoted by epicutaneous exposure to NNK (see Fig E2). Dexamethasone treatment significantly decreased intranasal CRA-induced AHR (see Fig E2, *A*) accompanied by a decrease in the magnitude of inflammation (eg, leukocyte infiltration and cytokine expression; see Fig E2, *B-E*). Epicutaneous NNK exposure could counter the effect of dexamethasone treatment on reducing plasma levels of IFN-γ and IL-33. Nevertheless, expression of IFN-γ and IL-9 in both the lung and spleen, as elicited by means of intranasal CRA or intranasal CRA plus epicutaneous NNK exposure, was always resistant to dexamethasone treatment (see Fig E2, *C-E*).

Host expression of α7 nAChRs is required for epicutaneous NNK exposure to exacerbate CRA-induced asthma pathology

Based on the finding that either NNK or the α7 nAChR agonist PNU282987 could exacerbate CRA-induced airway pathology, we assessed the extent to which the ability of epicutaneous exposure to NNK to exacerbate asthma pathology required expression of α7 nAChRs. We found that the features of CRA-induced asthma pathology that were elicited in α7

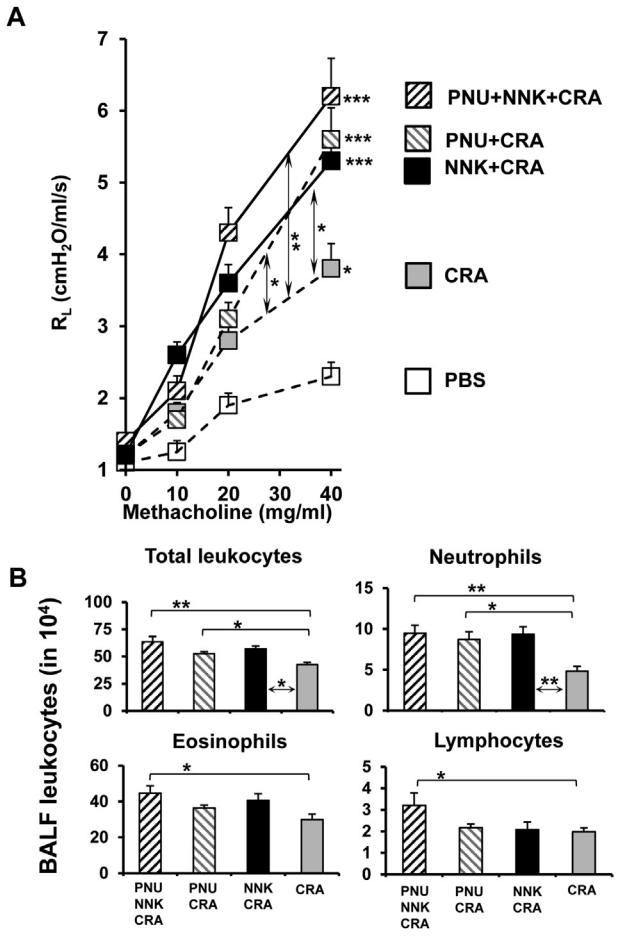

FIG 1. NNK or the α7 nAChR agonist PUN282987 can exacerbate AHR and airway inflammation in a CRA-induced mouse model of asthma. **A,** AHR to methacholine (assessed as R_L) measured 24 hours after the last intranasal challenge with antigen (CRA) or PBS in WT C57BL/6J mice. Results for Intraperitoneal PNU282987 (*PNU*) or epicutaneous NNK alone or PNU (intraperitoneal) and NNK (epicutaneous) 5 minutes before intranasal CRA on days 1, 2, 15, 18, and 21 are shown. **B,** Total BALF leukocytes, neutrophils, eosinophils, or lymphocytes 24 hours after the last intranasal challenge with CRA or PBS. N = 5 to 6 mice per group. $^*P < .05$, $^{**}P < .01$, or $^{***}P < .001$ versus corresponding PBS-treated controls (Fig 1, *A*) or the group indicated (Fig 1, *A* and *B*). In Fig 1, *B*, $P < .05$, .01, or .001 between the PBS-treated control group (not presented in the figures) versus the corresponding groups treated with CRA, PNU plus CRA, or NNK plus CRA. To simplify data presentation (in Fig 1, *A*), the PBS group depicted includes 3 randomly selected mice from each of the intranasal PBS control groups (in the presence or absence of treatment with NNK, PNU, or both) in these experiments. There were no statistically significant differences in responses observed in each of these PBS-treated control groups.

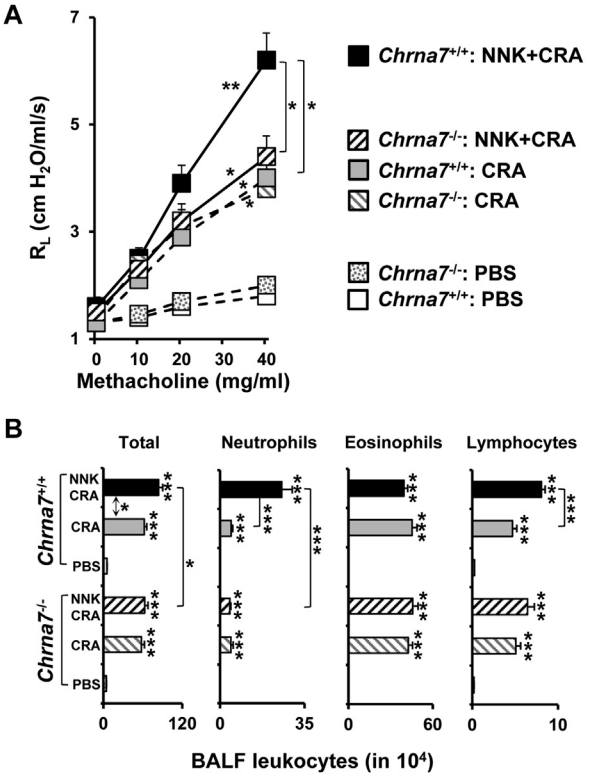

FIG 2. α7 nAChRs are required for epicutaneous NNK-dependent exacerbation of CRA-induced AHR and increases in BALF leukocyte counts but not for these features of CRA-induced asthma pathology induced in the absence of NNK. **A,** AHR to methacholine (assessed as R_L) measured 24 hours after the last intranasal challenge with CRA or PBS in WT C57BL/6J mice or α7 nAChR–deficient B6J-*Chrna7*$^{-/-}$ mice. **B,** Numbers of leukocytes in BALF recovered from mice 24 hours after the last intranasal administration of CRA or PBS. N = 5 to 6 mice per group. $^*P < .05$, $^{**}P < .01$, or $^{***}P < .001$ versus the corresponding PBS-treated controls or the group indicated.

nAChR–deficient B6J-*Chrna7*$^{-/-}$ mice were very similar to those observed in the identically treated WT mice, including AHR to methacholine; increased numbers of total bronchoalveolar lavage (BAL) leukocytes and BAL neutrophils, eosinophils, and lymphocytes; and interstitial infiltration of neutrophils and eosinophils in the lung (Figs 2 and 3), as well as increased numbers of goblet cells in the airway epithelium (ie, goblet cell hyperplasia) and increased lung collagen content (and subepithelial collagen deposition, Fig 3). However, epicutaneous exposure to NNK, which significantly exacerbated all of these features of asthma pathology in CRA-challenged WT mice, was essentially without effect in CRA-challenged B6J-*Chrna7*$^{-/-}$ mice (Figs 2 and 3). These findings indicate that host expression of α7 nAChRs is required for epicutaneous application of NNK to exacerbate AHR, airway inflammation, and airway remodeling in our CRA-induced asthma model.

MCs contribute to multiple features of this CRA-induced asthma model

In B6-*Cpa3-Cre; Mcl-1*$^{fl/fl}$ (H*K*) or B6J-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, both of which are markedly deficient in MCs, intranasal CRA challenge did not elicit AHR to methacholine but did induce slightly but significantly increased numbers of total BALF leukocytes and BALF neutrophils, eosinophils, and lymphocytes versus PBS-treated control valuess; however, the magnitude of such increases in MC-deficient mice was significantly lower than that in corresponding CRA-treated B6-*Cpa3-Cre; Mcl-1*$^{+/+}$ or B6J mice (Fig 4, *A* and *B*, and see Fig E3 in this article's Online Repository at www.jacionline.org). Moreover, the levels of CRA-induced lung infiltration by neutrophils and eosinophils, as well as airway goblet cell hyperplasia and enhanced lung collagen deposition, in MC-deficient H*K* mice were significantly lower than those exhibited in MC-containing B6-*Cpa3-Cre; Mcl-1*$^{+/+}$ mice (Figs 5 and 6). However, MC-deficient H*K* or B6J-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice that had been selectively engrafted with WT B6J MCs

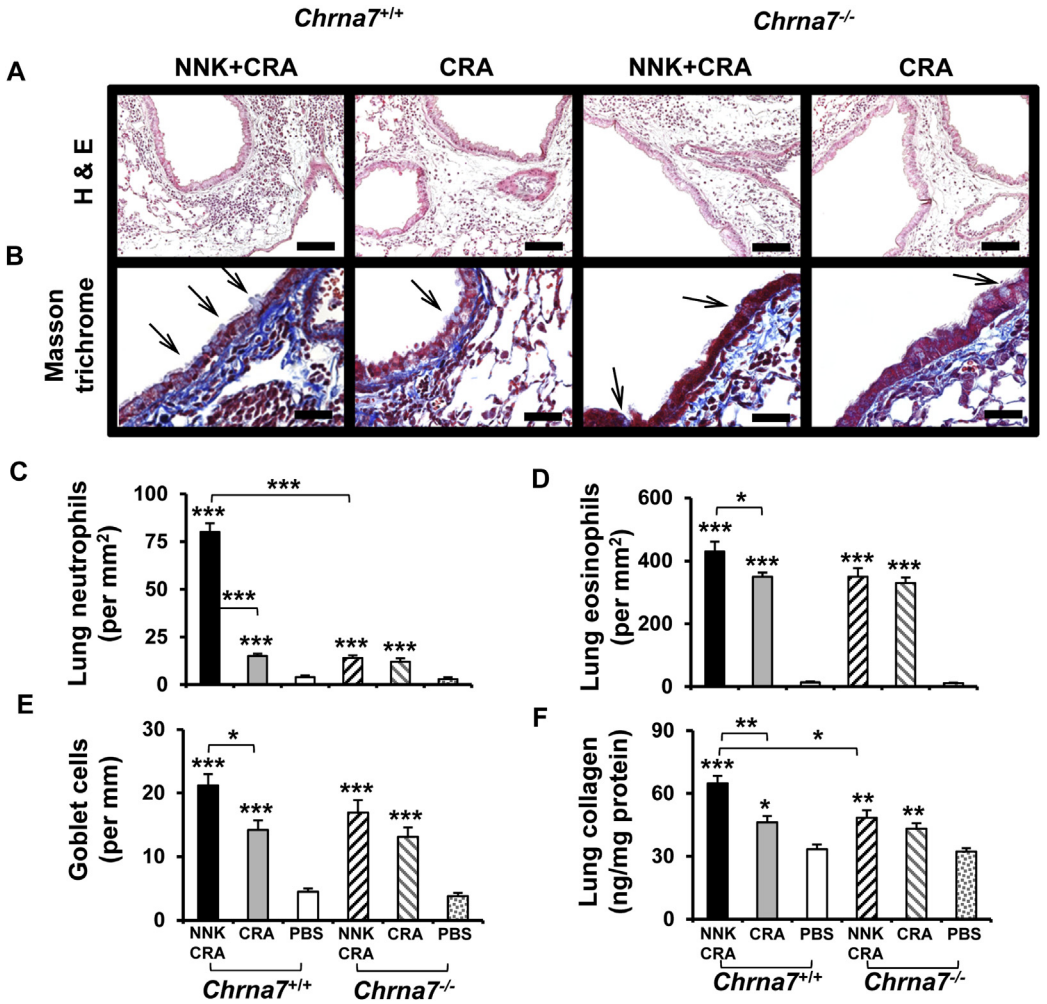

FIG 3. α7 nAChRs are required for epicutaneous NNK-dependent exacerbation of features of CRA-induced airway inflammation and remodeling. **A** and **B,** Hematoxylin and eosin *(H&E)*–stained (Fig 3, *A*) and Masson trichrome–stained (Fig 3, *B*) tissue sections of lungs demonstrating peribronchial or perivascular inflammatory infiltrates and mucus-secreting goblet cells (blue, some indicated with *arrows*) and subepithelial fibrosis (blue-stained collagen) 24 hours after the last intranasal CRA challenge. **C** and **D,** Numbers of neutrophils and eosinophils in lungs. **E,** Numbers of goblet cells along the airway epithelium. **F,** Lung collagen levels. N = 5 to 6 mice per group. $^{*}P < .05$, $^{**}P < .01$, or $^{***}P < .001$ versus corresponding PBS-treated controls or the group indicated. *Scale bars* = 50 μm (Fig 3, *A*) or 30 μm (Fig 3, *B*).

(BMCMCs or "MCs"; B6J-*Charma7*$^{+/+}$ MCs→H*K* or B6J-*Charma7*$^{+/+}$ MCs→*Kit*$^{\text{W-sh/W-sh}}$ mice) exhibited AHR to methacholine and features of allergic airway inflammation and airway wall remodeling that were statistically indistinguishable from those observed in the corresponding WT mice (Figs 4-6 and see Fig E3). Therefore, based on analyses in 2 types of MC-deficient mice whose MC deficiency reflects different genetic mechanisms, the development of these phenotypic features of asthma in this mouse model appeared to be largely MC dependent.

MC expression of both the FcεRI γ chain and α7 nAChRs is required for epicutaneous NNK exposure to exacerbate CRA-induced asthma pathology

MCs can be activated to release cytokines and other inflammatory mediators in response to the engagement of bivalent or multivalent antigen by antigen-specific IgE bound to IgE receptors (FcεRIs) on the MC surface. Moreover, it has been reported that such IgE-dependent MC mediator production can be reduced by exposure of MCs to NNK during the activation process.[24-26]

To assess the potential *in vivo* relevance of this finding, we investigated whether NNK could enhance features of asthma pathology in B6-*Fcer1g*$^{-/-}$ MCs→B6J-*Kit*$^{\text{W-sh/W-sh}}$ mice (MC-deficient *Kit*$^{\text{W-sh/W-sh}}$ mice that had been selectively engrafted with BMCMCs from FcεRI γ chain–deficient *Fcer1g*$^{-/-}$ mice), in which MCs lack the signaling γ chain of the FcεRI. In such mice MCs cannot be activated by FcεRI-dependent mechanisms (eg, by specific antigen and antigen-specific IgE). We found that B6-*Fcer1g*$^{-/-}$ MCs→B6J-*Kit*$^{\text{W-sh/W-sh}}$ mice subjected to our CRA-induced model of asthma exhibited little or no AHR to methacholine and had significantly lower numbers of BALF leukocytes after CRA challenge than did the corresponding WT (*Fcer1g*$^{+/+}$) BMCMC-engrafted mice (ie, MC-deficient

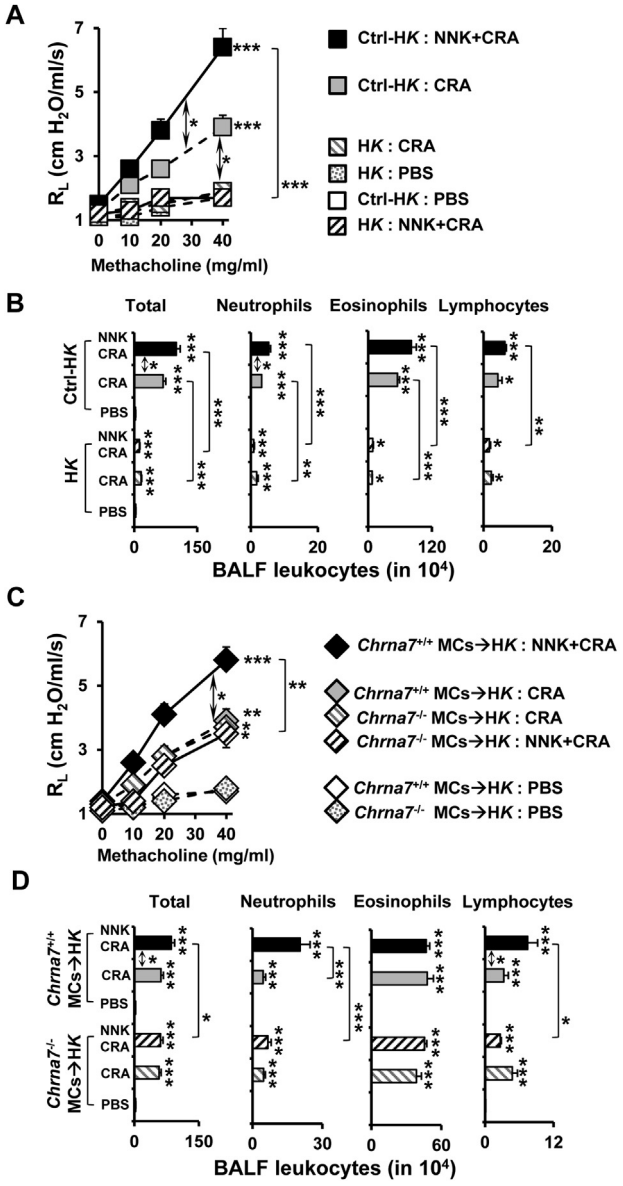

FIG 4. MCs and MC expression of α7 nAChRs are required for epicutaneous NNK-dependent exacerbation of CRA-induced AHR and increases in numbers of BALF leukocytes. **A** and **C,** AHR to methacholine (assessed as R_L) measured 24 hours after the last intranasal challenge with CRA or PBS in B6-*Cpa3-Cre; Mcl-1*$^{+/+}$ mice (MC-sufficient *[Ctrl-HK]*), MC-deficient B6-*Cpa3-Cre; Mcl-1*$^{fl/fl}$ mice *(HK)*, or B6-*Cpa3-Cre; Mcl-1*$^{fl/fl}$ mice engrafted with BMCMCs from WT B6J (*Chrna7*$^{+/+}$) mice versus α7 nAChR–deficient (B6J-*Chrna7* $^{-/-}$) mice (*Chrna7*$^{+/+}$ MCs→H*K* mice vs *Chrna7*$^{-/-}$ MCs→H*K* mice). **B** and **D,** Numbers of leukocytes in BALF recovered from mice 24 hours after the last intranasal administration of CRA or PBS. **P* < .05, ***P* < .01, or ****P* < .001 versus corresponding PBS-treated controls or the group indicated. N = 5 to 6 mice per group.

Kit$^{W\text{-}sh/W\text{-}sh}$ mice that had been selectively engrafted with BMCMCs from WT *Fcer1g*$^{+/+}$ mice; see Fig E4 in this article's Online Repository at www.jacionline.org). Numbers of lung MCs in the *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice engrafted with *Fcer1g*$^{-/-}$ were significantly lower than those in the *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice engrafted with *Fcer1g*$^{+/+}$ BMCMCs (see Fig E4, *C*). These findings indicate that expression of the FcεRI γ chain by the engrafted MCs was required for full development of the increase in lung MC numbers observed in the CRA-induced asthma model. This observation is consistent with similar findings reported by us in a chronic model of asthma induced by the antigen ovalbumin (OVA).[49,50]

Notably, epicutaneous exposure of CRA-challenged *Fcer1g*$^{-/-}$ MCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice to NNK neither enhanced airway responses to methacholine nor increased numbers of total (or individual types of) BALF leukocytes versus values in mice treated with CRA alone, whereas such effects of NNK exposure were observed in the *Fcer1g*$^{+/+}$ MCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice subjected to CRA challenge (see Fig E4). NNK exposure also had little or no effect on numbers of lung MCs in either CRA-challenged *Fcer1g*$^{-/-}$ MCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice or CRA-challenged *Fcer1g*$^{+/+}$ MCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. Taken together, our findings establish that the effects of epicutaneous exposure to NNK in enhancing airway responses to methacholine or numbers of BALF leukocytes in CRA-sensitized and challenged, MC-engrafted, genetically MC-deficient mice require that the engrafted MCs express the FcεRI γ chain.

We then assessed the contribution of MC expression of α7 nAChRs to NNK-dependent exacerbation of CRA-induced asthma pathology in the presence of intact antigen/IgE/FcεRI signaling. Specifically, we investigated the extent to which epicutaneous exposure to NNK could exacerbate features of asthma in MC-deficient H*K* mice that had been engrafted with BMCMCs from WT B6J (*Chrna7*$^{+/+}$) versus B6J-*Chrna7*$^{-/-}$ mice (ie, *Chrna7*$^{+/+}$ MCs→H*K* mice versus *Chrna7*$^{-/-}$ MCs→H*K* mice) and compared the results with those in MC-sufficient B6-*Cpa3-Cre; Mcl-1*$^{+/+}$ mice or MC-deficient H*K* mice. As expected, repeated CRA challenge of B6-*Cpa3-Cre; Mcl-1*$^{+/+}$ or *Chrna7*$^{+/+}$ MCs→H*K* mice induced characteristic features of asthma, including significant increases in AHR to methacholine and numbers of leukocytes in BALF (Fig 4, *C* and *D*), numbers of lung neutrophils and eosinophils, and numbers of airway goblet cells and lung collagen content (Figs 5 and 6). Except for numbers of BALF eosinophils, values for each of these features in CRA-challenged *Chrna7*$^{+/+}$ MCs→H*K* mice were significantly increased by means of epicutaneous exposure to NNK (Figs 4, *C* and *D*; 5; and 6). By contrast, although the features of CRA-induced asthma pathology in *Chrna7*$^{-/-}$ MCs→H*K* mice were similar to those in the corresponding *Chrna7*$^{+/+}$ MCs→H*K* mice, these features were not exacerbated further in mice also exposed epicutaneously to NNK exposure (Figs 4, *C* and *D*; 5; and 6).

These findings show that this NNK exposure protocol cannot significantly exacerbate the features of our CRA-induced asthma model in MC-engrafted H*K* mice in which the engrafted MCs are selectively deficient in α7 nAChRs. In addition, our findings also show that MC expression of α7 nAChRs was necessary for exposure to NNK to promote increased production of antigen-specific IgE/IgG$_1$, as well as increases in plasma levels of IFN-γ, IL-5, IL-9, and IL-33 (see Fig E5 in this article's Online Repository at www.jacionline.org).

Evidence that expression of α7 nAChRs exclusively in MCs is sufficient for epicutaneous NNK exposure to induce exacerbation of the CRA-induced asthma pathology

Our experiments in mice globally deficient in α7 nAChRs indicated that α7 nAChRs are required for epicutaneous exposure to NNK to exacerbate the features of the CRA-induced asthma

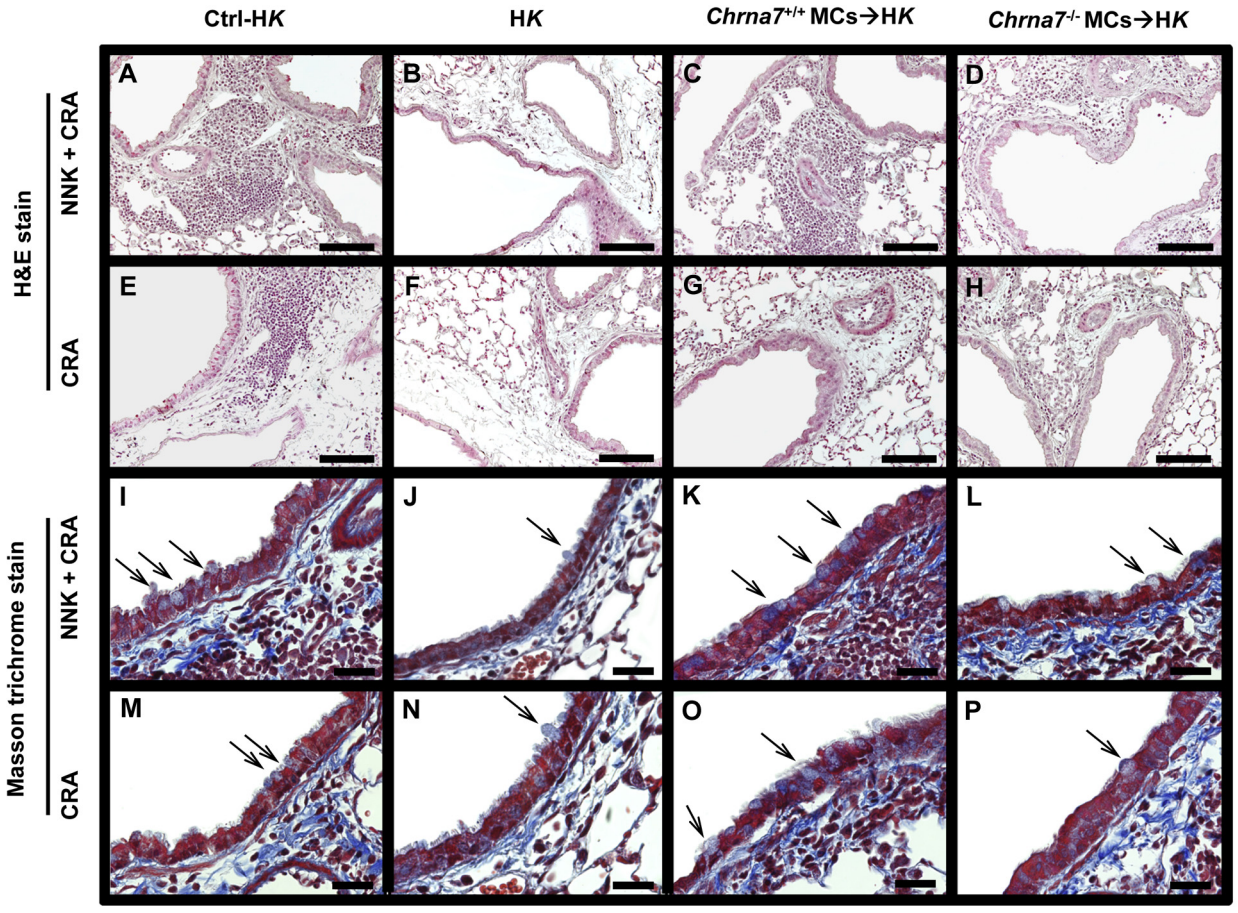

FIG 5. MCs and MC expression of α7 nAChRs are required for epicutaneous NNK-dependent exacerbation of features of CRA-induced airway inflammation and remodeling. **A-H,** Hematoxylin and eosin *(H&E)*–stained tissue sections demonstrating peribronchial or perivascular inflammatory infiltrates in the lungs 24 hours after the last intranasal challenge with CRA. **I-P,** Masson trichrome–stained tissue sections demonstrating mucus-secreting goblet cells along the airway epithelium (blue, some indicated with *arrows*) and subepithelial fibrosis (blue-stained collagen) in the lungs 24 hours after the last intranasal challenge with CRA. Tissues were collected from B6-*Cpa3-Cre; Mcl-1*$^{+/+}$ (MC-sufficient *[Ctrl-HK]*) mice, MC-deficient B6-*Cpa3-Cre; Mcl-1*$^{fl/fl}$ *(HK)* mice, or B6-*Cpa3-Cre; Mcl-1*$^{fl/fl}$ mice engrafted with BMCMCs from WT B6J mice (*Chrna7*$^{+/+}$ MCs→H*K* mice) versus α7 nAChR–deficient (B6J-*Chrna7*$^{-/-}$) mice (*Chrna7*$^{-/-}$ MCs→H*K* mice) subjected to intranasal antigen CRA challenge or to epicutaneous exposure to NNK and intranasal CRA challenge (NNK + CRA). *Scale bars* = 50 μm (Fig 5, *A-H*) or 20 μm (Fig 5, *I-P*).

model (Figs 2 and 3), and we provided evidence that α7 nAChR expression by MCs is required also (Fig 4, *C* and *D*; Figs 5 and 6). However, many cells in addition to MCs can express α7 nAChRs,[23-26,41,42] and we wondered whether non-MCs that bear α7 nAChRs can be needed also for epicutaneous exposure to NNK to exacerbate features of the CRA-induced asthma model. We addressed this question by performing experiments in mice in which MCs were the only cell that expressed α7 nAChRs. Specifically, we generated B6J-*Chrna7*$^{-/-}$-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice that lacked both MCs and α7 nAChRs and then engrafted these mice with either wild type B6J-*Chrna7*$^{+/+}$ BMCMCs (B6J-*Chrna7*$^{+/+}$ MCs→B6J-*Chrna7*$^{-/-}$-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, in which the engrafted WT *Chrna7*$^{+/+}$ MCs were the only cellular source of α7 nAChRs) or *Chrna7*$^{-/-}$ MCs (B6J-*Chrna7*$^{-/-}$ MCs→B6J-*Chrna7*$^{-/-}$-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, which remained globally deficient in α7 nAChRs but had both MCs and an intact antigen/IgE/FcεRI signaling pathway).

We found that intranasal CRA induced features of allergic airway inflammation and airway remodeling in both B6J-*Chrna7*$^{+/+}$ MCs→B6J-*Chrna7*$^{-/-}$-*Kit*$^{W\text{-}sh/W\text{-}sh}$ and B6J-*Chrna7*$^{-/-}$ MCs→B6J-*Chrna7*$^{-/-}$-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice; however, only CRA-challenged B6J-*Chrna7*$^{+/+}$ MCs→B6J-*Chrna7*$^{-/-}$-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice exhibited significant enhancement of asthma features when exposed to epicutaneous NNK. Specifically, in B6J-*Chrna7*$^{+/+}$ MCs→B6J-*Chrna7*$^{-/-}$-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, NNK exposure resulted in significant increases versus results in mice challenged with CRA alone in AHR to methacholine; numbers of BALF total leukocytes, neutrophils, and lymphocytes; lung tissue neutrophils and eosinophils; and airway epithelial goblet cells and in levels of lung collagen (Figs 7 and 8). However, when the CRA-induced asthma model was elicited in NNK-treated B6J-*Chrna7*$^{+/+}$ MCs→B6J-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, in which α7 nAChRs were expressed in MCs and other cell types, we detected significantly greater numbers of BALF total leukocytes and neutrophils and lung tissue neutrophils versus results in the corresponding NNK- and CRA-treated B6J-*Chrna7*$^{+/+}$ MCs→B6J-*Chrna7*$^{-/-}$-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (Figs 7 and 8). These 2 types of MC-engrafted mice (B6J-*Chrna7*$^{+/+}$

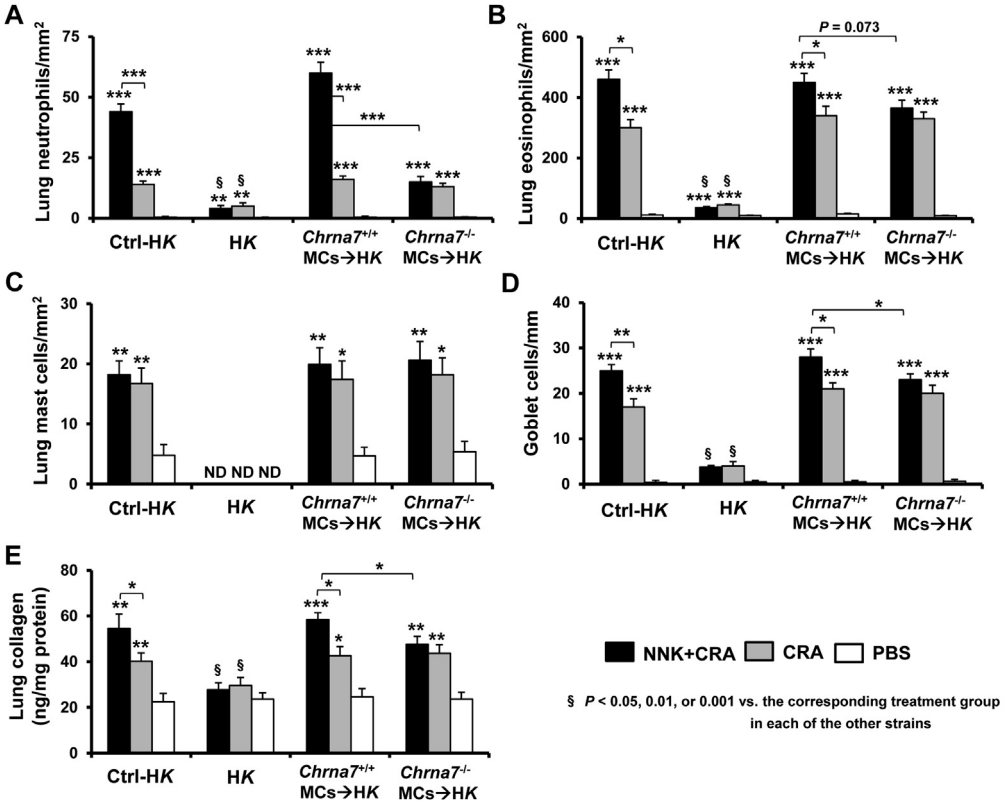

FIG 6. Influence of MCs and MC expression of α7 nAChRs on exacerbation of lung inflammation, goblet cell hyperplasia, and lung collagen deposition induced by epicutaneous exposure to NNK. **A-C,** Numbers of neutrophils, eosinophils, and MCs in the lungs. **D-E,** Numbers of goblet cells along the airway epithelium and levels of lung collagen. Lung tissues were collected 24 hours after the last intranasal challenge with CRA or PBS from B6-*Cpa3-Cre; Mcl-1*$^{+/+}$ (MC-sufficient *[Ctrl-HK]*) mice, MC-deficient B6-*Cpa3-Cre; Mcl-1*$^{fl/fl}$ *(HK)* mice, or B6-*Cpa3-Cre; Mcl-1*$^{fl/fl}$ mice selectively engrafted with BMCMCs from WT B6J-(*Chrna7*$^{+/+}$) versus α7 nAChR–deficient (B6J-*Chrna7*$^{-/-}$) mice (*Chrna7*$^{+/+}$ MCs→H*K* vs *Chrna7*$^{-/-}$ MCs→H*K* mice). **P* < .05, ***P* < .01, or ****P* < .001 versus the corresponding PBS-treated controls or the group indicated. §*P* < .05, .01, or .001 between the specific treatment group in the H*K* strain versus the corresponding treatment group in each of the other strains of mice. *ND,* Not detected. N = 5 to 6 mice per group.

MCs→B6J-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice or B6J-*Chrna7*$^{+/+}$ MCs→B6J-*Chrna7*$^{-/-}$-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice) treated with NNK plus CRA did not exhibit significant differences in the numbers of MCs engrafted in the lungs (Fig 8, *C*). In aggregate, these findings suggest that NNK-dependent exacerbation of asthma pathology in this CRA model is largely dependent on MC expression of α7 nAChRs but that other α7 nAChR-bearing cells also can contribute to at least some of the findings.

Taken together, our observations indicate that the effects of NNK on α7 nAChRs expressed by FcεRIγ-expressing MCs contribute significantly to the ability of epicutaneous exposure to NNK to exacerbate the severity of multiple features of the asthma pathology elicited in mice by the clinically relevant antigen CRA.

DISCUSSION

In this study we established a mouse model of asthma based on repeated intranasal challenges with the clinically relevant antigen CRA. This CRA-induced model exhibits several major features of asthma, including AHR to methacholine, airway inflammation, and airway wall remodeling. Using this model, we identified an unexpected mechanism by which epicutaneous exposure to NNK, a major component of THS, can exacerbate several features of asthma pathology. Specifically, we found that epicutaneous exposure to NNK (for which the α7 nAChR is the high-affinity receptor[13]) can enhance AHR to methacholine, airway inflammation, and airway wall remodeling in the CRA-induced model and that for NNK to do this requires that MCs express both α7 nAChRs and functionally active FcεRIs. We also found that epicutaneous exposure to NNK can exacerbate certain features of asthma pathology in a different mouse model, that induced by OVA in the absence of artificial adjuvant (see Fig E6 in this article's Online Repository at www.jacionline.org).

Our results are unexpected in part because a number of studies have suggested that nicotine or other agonists of nAChRs can activate an α7 nAChR–dependent anti-inflammatory mechanism (reviewed by de Jonge and Ulloa[51]), findings that contributed to a growing body of evidence showing that the central nervous system can modulate components of the immune system.[52] Indeed, the so-called "cholinergic anti-inflammatory pathway" and its potential to influence immune responses and inflammatory cascades have attracted substantial interest because of their obvious relevance to a variety of debilitating human diseases.[51,53,54] However, there is conflicting evidence about the importance of this pathway *in vivo*. In some studies nicotine or nicotinic agonist

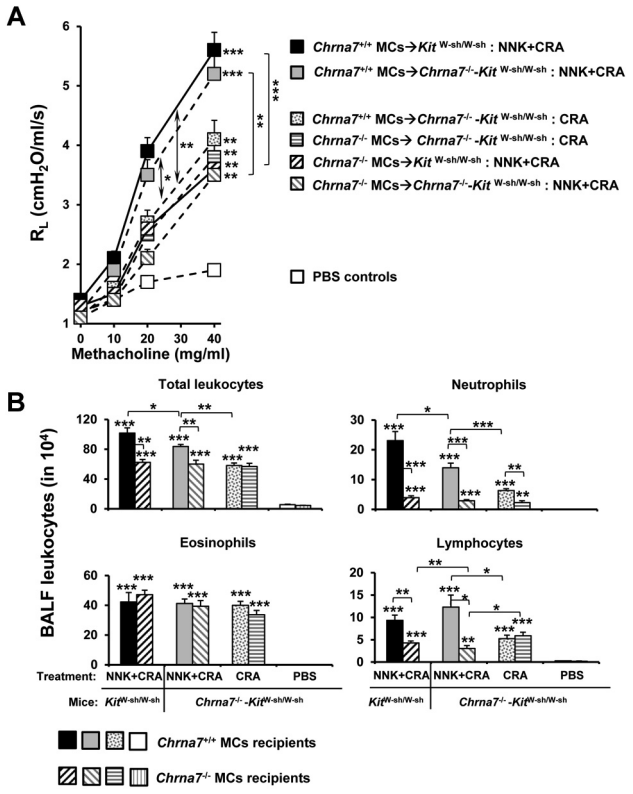

FIG 7. Influence of MCs or global expression of α7 nAChRs on exacerbation of AHR and airway inflammation by epicutaneous exposure to NNK. **A,** AHR (assessed as R_L) measured 24 hours after the last intranasal challenge with CRA or PBS in MC-deficient B6J-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice engrafted with BMCMCs from B6J (*Chrna7*$^{+/+}$) versus α7 nAChR–deficient (B6J-*Chrna7*$^{-/-}$) mice (*Chrna7*$^{+/+}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ vs B6J-*Chrna7*$^{-/-}$ BMCMCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice) or mice deficient in both MCs and α7 nAChRs (B6J-*Chrna7*$^{-/-}$-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice) engrafted with BMCMCs from B6J (*Chrna7*$^{+/+}$) versus α7 nAChR–deficient (B6J-*Chrna7*$^{-/-}$) mice (*Chrna7*$^{+/+}$ BMCMCs→*Chrna7*$^{-/-}$-*Kit*$^{W\text{-}sh/W\text{-}sh}$ vs *Chrna7*$^{-/-}$ BMCMCs→*Chrna7*$^{-/-}$-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice). **B,** Numbers of total or individual types of leukocytes in BALF from mice 24 hours after the last intranasal CRA or PBS administration. **P* < .05, ***P* < .01, or ****P* < .001 versus corresponding PBS-treated controls or the group indicated. N = 5 to 6 mice per group. To simplify data presentation, the PBS group depicted in the graphs includes 3 randomly selected mice from the PBS control group of each strain of mice used in these experiments. There were no statistically significant differences in the responses observed in each of these PBS-treated control groups.

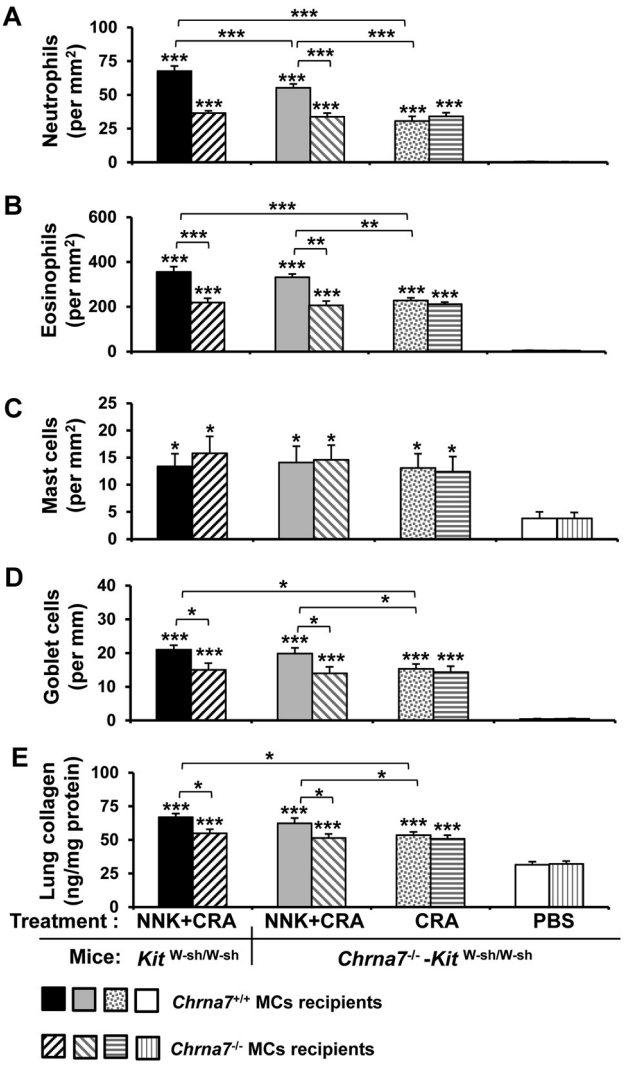

FIG 8. Influence of MCs or global expression of α7 nAChRs on exacerbation of lung inflammation, goblet cell hyperplasia, and lung collagen deposition through epicutaneous exposure to NNK. **A-C,** Numbers of neutrophils, eosinophils, and MCs in the lungs. **D-E,** Numbers of goblet cells in airway epithelium and levels of lung collagen. Lung tissues were collected 24 hours after the last intranasal challenge with CRA or PBS from MC-deficient B6J-*Kit*$^{W\text{-}sh/W\text{-}sh}$ (*Kit*$^{W\text{-}sh/W\text{-}sh}$) mice selectively engrafted with BMCMCs from WT B6J (*Chrna7*$^{+/+}$) versus α7 nAChR–deficient (B6J-*Chrna7*$^{-/-}$) mice or mice that lack both MCs and α7 nAChRs (B6J-*Chrna7*$^{-/-}$-*Kit*$^{W\text{-}sh/W\text{-}sh}$) that had been engrafted with BMCMCs from WT B6J (*Chrna7*$^{+/+}$) versus α7 nAChR–deficient (B6J-*Chrna7*$^{-/-}$) mice. **P* < .05, ***P* < .01, or ****P* < .001 versus the corresponding PBS-treated controls or the group indicated. N = 5 to 6 mice per group. To simplify data presentation, the PBS group depicted in the graphs includes 3 randomly selected mice from the PBS control group for each strain of mouse that had been engrafted with the same strain of BMCMCs used in these experiments. There were no statistically significant differences in the responses observed in each of these PBS-treated control groups.

were found to reduce lung inflammation, AHR, and levels of antigen-specific IgE in mouse models significantly,[55,56] observations supporting an anti-inflammatory role for nicotine and cholinergic pathways. By contrast, Mishra et al[41] reported that although systemic administration of nicotine strongly diminished lung T_H2 responses in antigen-challenged rats, which had been sensitized with ragweed or house dust mite antigen plus aluminum hydroxide, nicotine treatment neither reduced β-hexosaminidase levels in the BALF nor diminished methacholine-induced airway resistance *in vivo* but significantly increased airway intraepithelially stored mucus and expression of Muc5ac mRNA. The latter study indicates that nicotine treatment can decrease, increase, or have no significant effects on various features of rodent models of asthma.

In our CRA-induced mouse model of asthma, we detected no evidence for the NNK-dependent activation of a cholinergic anti-inflammatory pathway that can mitigate the inflammatory response. To the contrary, we found that in this CRA-induced model of asthma, intraperitoneal administration of the α7 nAChR agonist PNU282987 or epidermal application of NNK promoted the development of AHR and airway inflammation (Fig 1). Subsequent experiments were consistent with this finding and provided evidence for the important role of MCs and MC expression of α7 nAChRs in contributing to the ability of exposure to NNK to exacerbate asthma pathology in this model. Notably,

our mouse model of asthma is based on use of an allergen that is clinically relevant in that CRA has been reported to be the most important allergen for eliciting asthma in inner-city households in the United States.[44-46]

Although we performed these experiments mainly using B6-H*K* mice (which are genetically deficient in MCs through a mechanism that does not depend on abnormalities in c-*Kit* or its expression), we found that similar results were obtained in experiments using either H*K* mice or B6J-*Kit*$^{W\text{-}sh/W\text{-}sh}$ genetically MC-deficient mice as recipients of transfer of WT or genetically altered BMCMCs (Fig 4 and see Fig E3). This encouraged us to use B6J-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice to generate mice that both essentially lack MCs and are globally deficient in α7 nAChRs. These mice permitted us to show that expression of α7 nAChRs solely in adoptively transferred MCs is sufficient for epicutaneous exposure to NNK to enhance multiple features of our CRA-induced asthma model in MC-engrafted B6J-*Chrna7*$^{-/-}$-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice.

It is well known that the children of smokers are at increased risk for asthma and for asthma exacerbations of asthma if the disease is already present.[57-60] Moreover, it has been proposed that cigarette smoking and exposure to secondhand smoke can contribute to worse outcomes and diminished effectiveness of steroid therapy in asthmatic patients.[61-63] Other evidence supports the idea that short- and long-term effects of tobacco smoke exposure can influence the structure of the developing lung, as well as lung physiology, effects that can have lifelong consequences,[64-67] and there is evidence that the most common preventable early-life risk factor for asthma is exposure to tobacco smoke.[68] Long-term exposure of mice to THS can induce pulmonary toxicity by itself.[9] However, our study indicates that in mice exposure to a component of THS, even at doses too low to induce obvious histologic effects on the lungs, can significantly exacerbate multiple features of asthma pathology. The extent to which our findings are relevant to human subjects exposed to both environmental allergens and THS remains to be determined, and *in vivo* studies like those we performed in mice could not be considered in human subjects because of obvious ethical concerns. However, our findings in this mouse model of asthma show that the effects on MCs of one component of THS can exacerbate pathologic features of asthma and raise the possibility that such a pathway can contribute to some of the adverse health effects of environmental exposure to nicotine, nicotine-derived nitrosamines, or both.

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Key messages

- **Skin exposure to the THS component NNK can exacerbate features of asthma pathology in a mouse model.**
- **MC expression of α7 nAChRs contributes to the ability of skin exposure to NNK to exacerbate asthma pathology in this model.**

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METHODS

MC engraftment

Engraftment of BMCMCs into MC-deficient B6-*Cpa3-Cre; Mcl-1*$^{fl/fl}$ or B6J-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice or into B6J-*Chrna7*$^{-/-}$-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (that lack both MCs and α7 nAChRs) was performed, as previously described,[E1,E2] with minor modifications. Bone marrow cells derived from 4-week-old female B6, B6-*Chrna7*$^{-/-}$ or B6-FcR1γ-deficient *Fcer1g*$^{-/-}$ mice were cultured in 20% WEHI-3–conditioned medium (ATCC no. TIB-68) as a source of IL-3 for 4 to 5 weeks to generate cell populations that contained more than 99% immature MCs. BMCMCs (2×10^6) were injected into each mouse through the tail vein, and the recipients (eg, B6 BMCMCs → *Kit*$^{W\text{-}sh/W\text{-}sh}$, B6-*Chrna7*$^{-/-}$ BMCMCs → *Kit*$^{W\text{-}sh/W\text{-}sh}$, B6 BMCMCs → *Cpa3-Cre; Mcl-1*$^{fl/fl}$, B6-*Chrna7*$^{-/-}$ BMCMCs → *Cpa3-Cre; Mcl-1*$^{fl/fl}$, B6 [*Chrna7*$^{+/+}$]-BMCMCs → *Chrna7*$^{-/-}$-*Kit*$^{W\text{-}sh/W\text{-}sh}$, B6 [*Chrna7*$^{-/-}$]-BMCMCs → *Chrna7*$^{-/-}$-*Kit*$^{W\text{-}sh/W\text{-}sh}$, or B6-*Fcer1g*$^{-/-}$ BMCMCs → *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice) were used for experiments 12 weeks later.

Airway challenge with CRA

Mice were subjected to intranasal administration of 10 μg of CRA (a mixture of whole-body extract of American cockroach *[Periplaneta americana]*; Greer Laboratories, Lenoir, NC) in 0.03 mL of PBS on days 1, 2, 15, 18, and 21; control mock-sensitized mice received intranasal challenges with PBS on the same schedule.

Epicutaneous exposure to NNK

Mice were subjected to epidermal painting on the inner side of the left ear pinna with NNK (Sigma-Aldrich, St Louis, Mo) at 0.5 mg/kg BW in 0.01 mL of ethanol on days 1, 2, 15, 18, and 21 in each instance 5 minutes before CRA challenge. Dermal painting of NNK (0.5 mg/kg BW in 0.01 mL of ethanol) did not induce ear swelling and vasodilation, which was confirmed by using the Evans blue test and measurement of ear thickness (Fig E1, *A* and *B*). Cell-culture experiments also revealed that NNK alone could not induce MC degranulation (Fig E1, *C*).

It has been reported that exposure of mice to THS (which has been shown to contain NNK[E3]) under conditions that mimicked exposure of human subjects can induce a proinflammatory environment in the lung, including infiltration of inflammatory cells and increased lung collagen (by using Masson trichrome stain). Therefore we performed preliminary experiments to define an amount of NNK that did not by itself induce histologically detectable lung inflammation. We found that 5 epicutaneous applications of NNK, consisting of 0.5 mg/kg BW NNK per treatment, did not elicit a histologically detectable inflammatory response, such as infiltration of inflammatory cells into the lung, nor did it increase levels of lung collagen, as assessed in Masson trichrome–stained sections (Fig E1, *D*).

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) is the principal metabolite of NNK. Urinary concentrations of NNAL can be measured to quantitatively estimate exposure to NNK.[E4] In the present study the urinary NNAL level measured 24 hours after the last epicutaneous NNK exposure was 1259 ± 221 pg/mL (614-2461, pg/mL). This measurement was performed at the Clinical Pharmacology Laboratory, University of California, San Francisco. The potential endotoxin contamination of NNK was excluded by means of examination with a Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Waltham, Mass). In addition, we confirmed the influence of epicutaneous NNK exposure on airway recruitment of neutrophils by excluding the influence of endotoxin. Specifically, we subjected TLR4-deficient *Tlr4*$^{-/-}$ mice to intranasal CRA challenge versus intranasal CRA challenge plus epicutaneous NNK exposure (Fig E7). This further demonstrated the significant similarity of the pattern of responses of *Tlr4*$^{-/-}$ versus WT *Tlr4*$^{+/+}$ mice either to intranasal CRA challenge or intranasal CRA challenge plus epicutaneous NNK exposure in the expression of the cytokines IFN-γ, IL-5, and IL-9 in the lungs and spleens (Fig E7, *B* and *C*).

Measurement of airway reactivity to methacholine

Invasive measurements of airway reactivity in anesthetized, tracheostomized, mechanically ventilated mice were performed 24 hours after the last intranasal exposure (eg, to CRA or PBS) by using the Mouse RC system (BUXCO Research Systems, Wilmington, NC). Aerosolized methacholine was administered in increasing concentrations (0, 10, 20, and 40 mg/mL), with individual doses separated by 2 minutes. R_L was continuously computed by fitting flow, volume, and pressure to an equation of motion for each aerosol challenge period, which consisted of a 0.5-minute aerosol exposure and a 1.5-minute period after exposure, as described previously.[E1,E2]

BAL and histology

For BAL, mice were killed by using CO_2 inhalation 24 hours after the last CRA or PBS challenge. The left lung was ligated and removed for protein extraction, whereas the right lung lobes were lavaged once with ice-cold HBSS (BAL) and then fixed (10% formaldehyde) and paraffin embedded. Paraffin sections of 5 μm were mounted on Superfrost Plus glass slides (Fisher Scientific) and stained with hematoxylin and eosin or Masson trichrome stain.

On hematoxylin and eosin–stained tissue sections, neutrophils and eosinophils were identified according to their morphologic features. At least 8 typical peribronchial regions (cross-sections from the basement membrane to the alveolar wall) per mouse were randomly selected in a blinded fashion for neutrophil/eosinophil counting in 6 high-power fields (×400 total magnification), which were located at the positions of 12, 2, 4, 6, 8, and 10 o'clock. MCs were identified in toluidine blue–stained tissue sections. At least 8 typical peribronchial regions were randomly selected, and numbers of MCs were counted in a blinded fashion throughout the entire peribronchial regions. The areas examined for neutrophils/eosinophils or for MC counting were measured by using the Image Pro Plus imaging software system (Media Cybernetics, Rockville, Md). Goblet cells were identified by using Masson trichrome staining. At least 8 typical bronchial cross-sections were randomly selected, and numbers of goblet cells along the bronchial epithelium were counted in a blind fashion. The length of the bronchial epithelium was measured with the Image Pro Plus imaging software system (Media Cybernetics).

Tissue protein extraction

Lung or spleen tissue protein was extracted by using the T-PER Tissue Protein Extraction Reagent purchased from Thermo Scientific (Rockford, Ill). Half of the left lung or one third of the spleen was placed into 1 mL of protein extraction reagent, followed by homogenization, and then the samples were centrifuged at 10,000*g* for 5 minutes to pellet cell/tissue debris; supernatants were collected for further analyses.

Protein, collagen, and cytokine measurements

Protein levels of tissue samples were measured by using a BCA Protein Assay Kit purchased form Thermo Scientific. Tissue collagen levels were measured with a Sircol Soluble Collagen Assay Kit purchased from Biocolor (Carrickfergus, United Kingdom). ELISA kits for measuring IFN-γ, IL-5, IL-9, and IL-33 were purchased from Thermo Fisher Scientific.

Airway challenge with OVA

Female C57BL/6 mice at 10 weeks of age were sensitized by means of 3 intraperitoneal injections of 50 μg of OVA (Sigma-Aldrich) per injection in 0.1 mL of PBS on days 1, 4, and 7. Starting on day 12, these mice were subjected to intranasal challenge with 20 μg of OVA/0.03 mL of PBS weekly 9 times (Fig E6, *A*); control mice received intraperitoneal injections and intranasal challenges with PBS on an identical schedule.[E1,E2]

Statistics

Differences in airway reactivity between the different groups were tested for statistical significance by using repeated ANOVA followed by the Fisher's protected least significant difference (PLSD) test (StatView, version 5.0.1). Unless otherwise specified, differences among experimental groups in the same strain of mice were assessed by using ANOVA, followed by Tukey-Kramer tests, whereas differences between groups of mice of different strains that were subjected to identical treatment were tested with the unpaired Student *t* test (2-tailed; GraphPad InStat, version 3.0.1; GraphPad Software, La Jolla, Calif). A *P* value of less than .05 was considered statistically significant. Unless otherwise specified, all data are presented as means ± SEMSs. Each experiment reported here has been repeated twice to confirm their reproducibility, which generated similar results.

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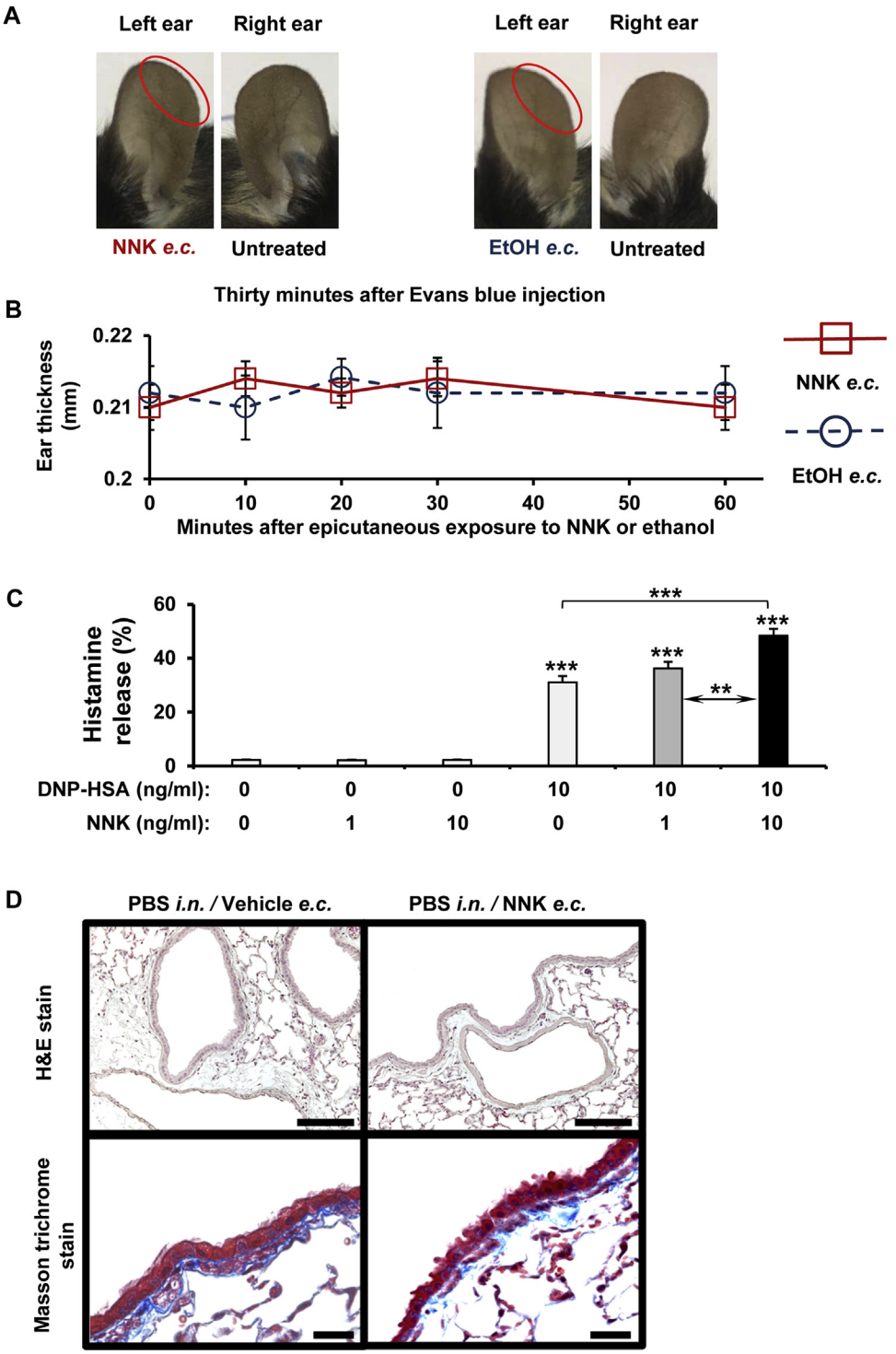


FIG E1. A, WT C57BL/6 (B6) mice were injected intravenously with 0.2 mL of Evans Blue dye (50 mg/kg BW in normal saline) and then painted epicutaneously *(e.c.)* with NNK (0.5 mg/kg BW/0.01 mL of ethanol) or ethanol (0.01 mL) on left ear skin *(red circle)*. Thirty minutes later, there was no NNK-induced increase in Evans Blue extravasation. **B,** Ear thickness of NNK-exposed mice measured at the site indicated with a *red circle* in Fig E1, *A*, with a dial thickness gauge (G-1 A, Ozaki, Japan) was not altered significantly compared with vehicle-painted control values. **C,** Culture supernatants of anti-DNP IgE–sensitized BMCMCs from C57BL/6 mice collected (for calculation of % histamine release) 1 hour after addition of DNP-HSA, NNK, or both. $^{**}P < .01$ or $^{***}P < .001$ versus each group generated without DNP-HSA stimulation (0 ng/mL) or versus the indicated group (data are from 3 independent experiments). **D,** Masson trichrome–stained lung sections in C57BL/6 mice subjected to epicutaneous *(e.c.)* NNK (0.5 mg/kg BW/0.01 mL of ethanol at days 1, 2, 15, 18 and 21 five minutes before intranasal *[i.n.]* PBS administration; *right*) versus those in mice subjected to epicutaneous exposure to ethanol and intranasal PBS administration alone (*left*; tissues collected 24 hours after the last intranasal PBS administration). *Scale bars* = 100 μm *(upper panels)* or 30 μm *(lower panels)*. *H&E*, Hematoxylin and eosin.

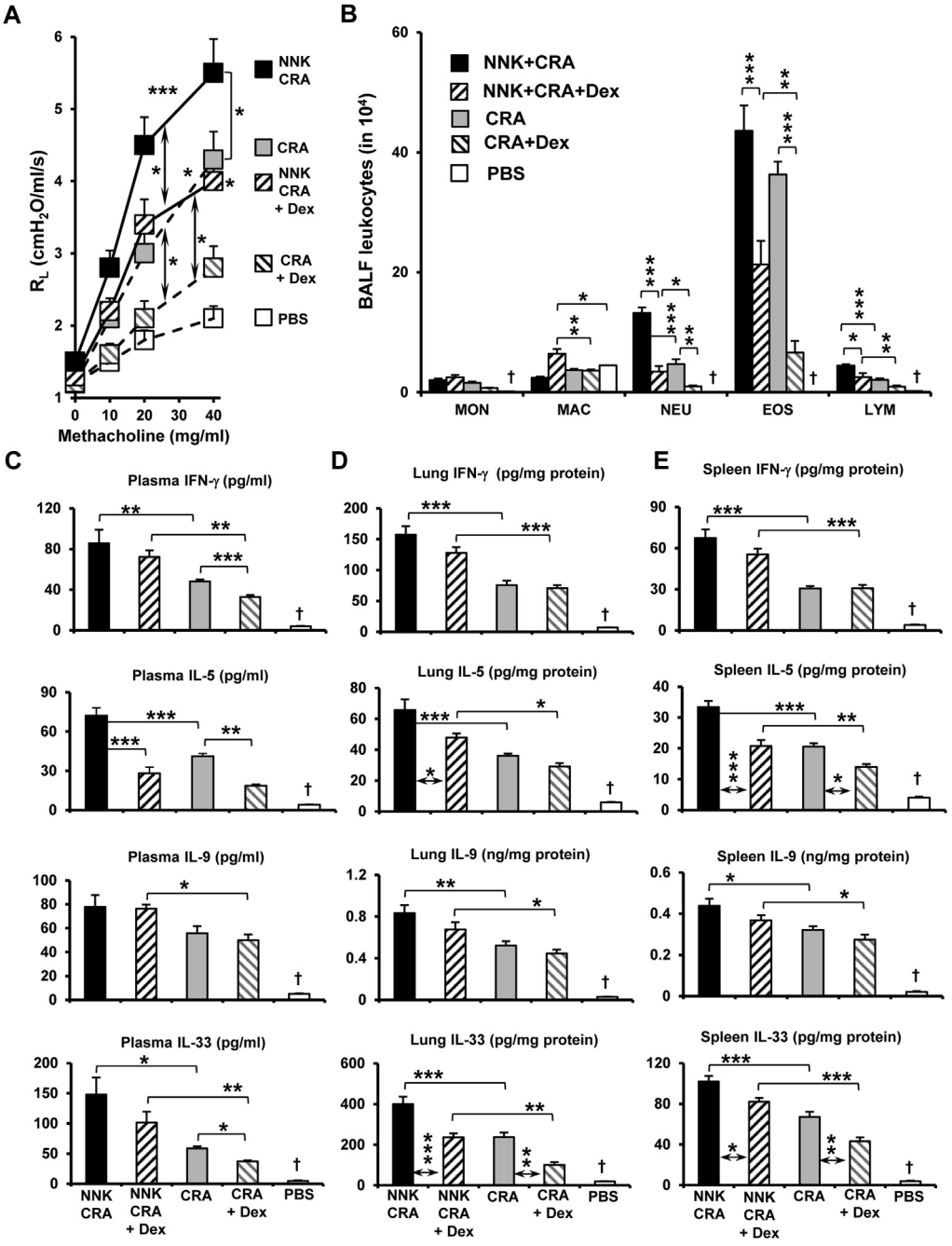


FIG E2. Influence of dexamethasone *(Dex)* treatment on development of characteristic features of allergic asthma in this mouse model. Asthma models elicited in C57BL/6 mice after intranasal CRA challenge in the presence or absence of epicutaneous *(e.c.)* NNK exposure were subjected to 2 dexamethasone treatments 30 minutes after CRA challenge at days 18 and 21, with a dose of 1 mg/kg BW (dexamethasone solubilized in normal saline for intraperitoneal injection). Dexamethasone treatment–induced changes in AHR to methacholine (assessed as R_L; **A**), profile of BALF-recovered leukocytes **(B)**, and levels of cytokines in the plasma **(C)**, lung **(D)**, and spleen **(E)** were determined 24 hours after the last intranasal CRA or PBS challenge. *EOS*, Eosinophils; *LYM*, lymphocytes; *MAC*, macrophages; *MON*, monocytes; *NEU*, neutrophils. $*P < .05$, $**P < .01$, or $***P < .001$ versus the corresponding PBS-treated controls (in Fig E2, *A*) or the group indicated (in Fig E2, *A-E*); †$P < .05$, .01, or .001 between the PBS control group versus the corresponding CRA-treated group (Fig E2, *B-E*). To simplify data presentation, the PBS group depicted in the graphs includes 3 randomly selected mice from each intranasal PBS-treated group in these experiments. There were no statistically significant differences in the responses observed in each of these PBS-treated control groups.

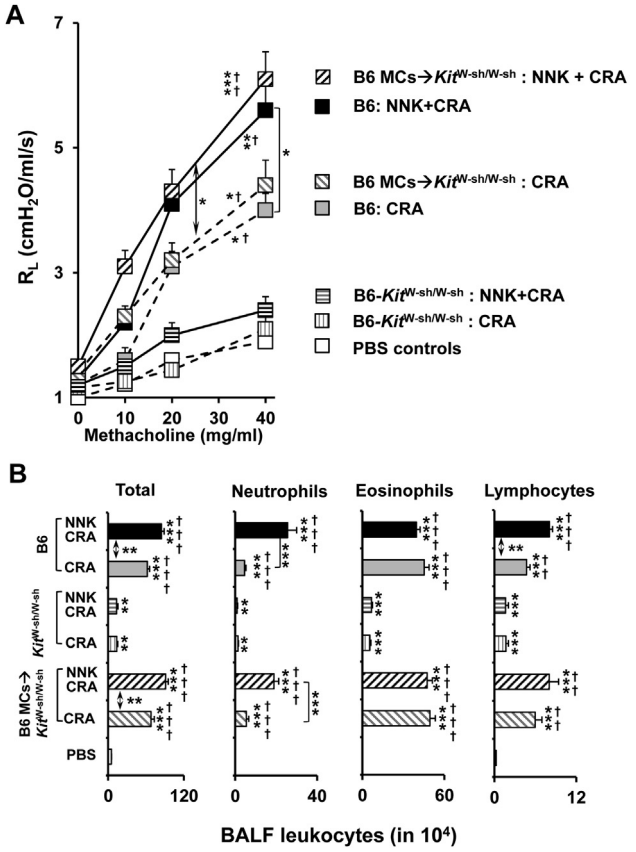


FIG E3. Influence of MCs and MC α7 nAChRs on exacerbation of AHR and airway inflammation by means of epicutaneous exposure to NNK. **A,** AHR to methacholine (assessed as R_L) measured 24 hours after the last intranasal challenge with CRA or PBS in WT C57BL/6J mice (B6J), MC-deficient B6J-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, or B6J-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice that had been selectively engrafted with BMCMCs from B6 mice (B6J BMCMCs → *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice). **B,** Numbers of individual types of leukocytes in BALF recovered from mice 24 hours after the last intranasal administration of CRA or PBS. **P* < .05, ***P* < .01, or ****P* < .001 versus the corresponding PBS-treated controls or the group indicated; †*P* < .05, ††*P* < .01, or †††*P* < .001 versus the corresponding group of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. N = 5 to 6 mice per group. To simplify data presentation, the PBS group depicted in the graphs includes 3 randomly selected mice from the PBS control group of each strain of mouse used in these experiments. There were no statistically significant differences in the responses observed in each of these PBS-treated control groups.

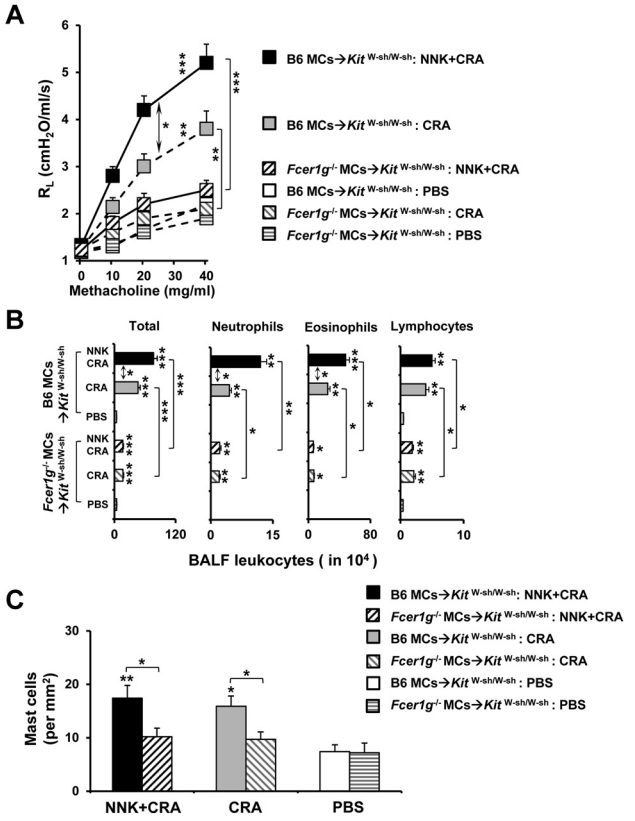
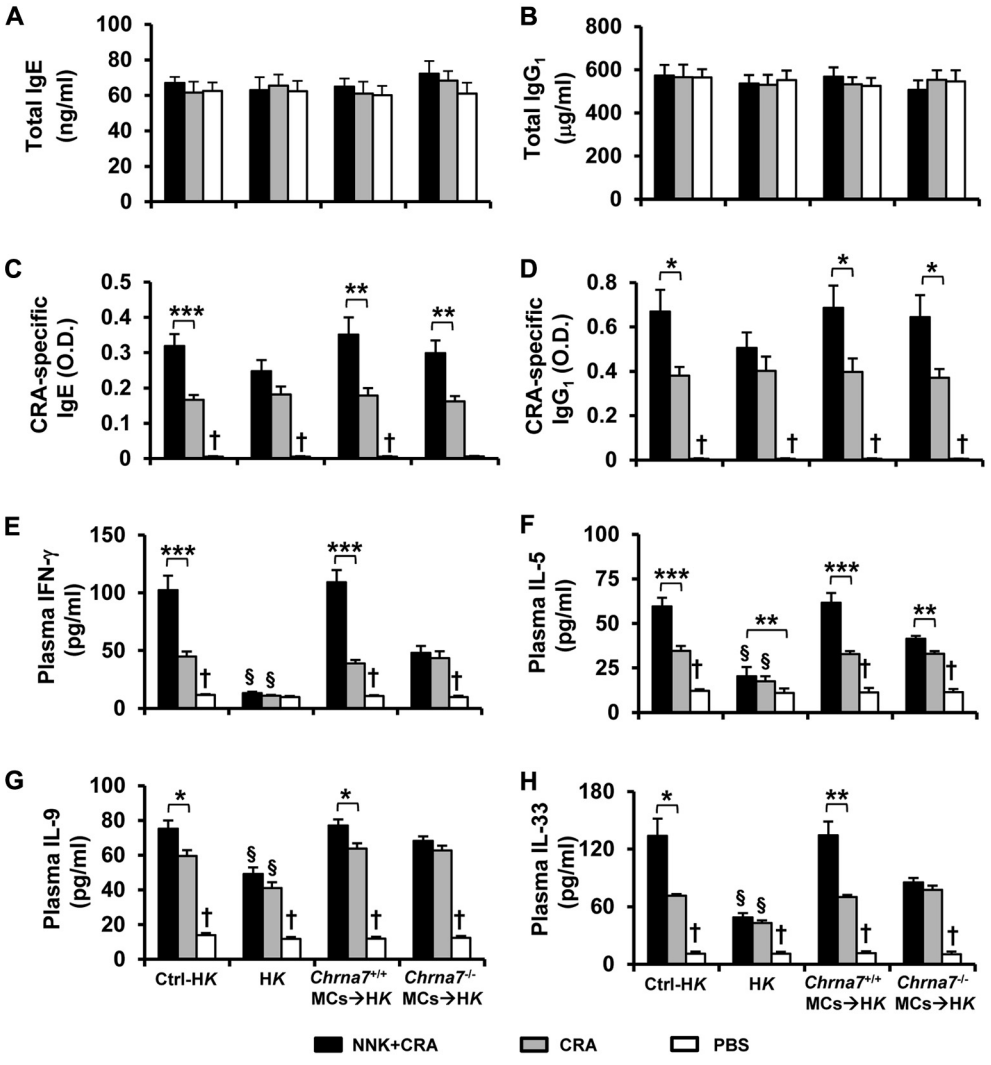


FIG E4. MC expression of FcεRIγ is required for full expression of CRA-induced AHR and increases in numbers of BALF leukocytes and also for exacerbation of numbers of BALF leukocytes in this model by means of epicutaneous exposure to NNK. **A,** AHR to methacholine (assessed as R_L) measured 24 hours after the last intranasal challenge with CRA or PBS in MC-deficient B6J-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice that had been selectively engrafted with BMCMCs from WT B6 (*Fcer1g*$^{+/+}$) versus FcεRIγ-deficient (*Fcer1g*$^{-/-}$) mice (B6 MCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$ versus *Fcer1g*$^{-/-}$ MCs→*Kit*$^{W\text{-}sh/W\text{-}sh}$). **B,** Numbers of individual types of leukocytes in BALF recovered from mice 24 hours after the last intranasal administration of CRA or PBS. **C,** Numbers of MCs in the lungs. **P* < .05, ***P* < .01, or ****P* < .001 versus corresponding PBS-treated control values or the group indicated. N = 5 to 6 mice per group.



† $P < 0.05$, 0.01, or 0.001 vs. NNK+CRA or CRA-treated group in the same strain;

§ $P < 0.05$, 0.01, 0.001 vs. the corresponding treatment group in each of the other strains.

FIG E5. Plasma levels of total IgE/IgG$_1$, antigen-specific IgE/IgG$_1$, and cytokines. Concentrations of total IgE/IgG$_1$, CRA-specific IgE/IgG$_1$, and cytokines were measured in WT, H*K*, or H*K* mice engrafted with BMCMCs from *Chrna7*$^{+/+}$ or *Chrna7*$^{-/-}$ mice 24 hours after the last intranasal CRA or PBS challenge in the presence or absence of epicutaneous NNK exposure. *$P < .05$, **$P < .01$, or ***$P < .001$ versus the group indicated. †$P < .05$, .01, or .001 between PBS controls versus groups of same strain of mice subjected either to intranasal CRA challenge or to intranasal CRA challenge plus epicutaneous NNK exposure. §$P < .05$, .01, or .001 between the H*K* mice versus each of the groups of mice of other strains after identical treatment with CRA or CRA plus NNK. N = 5 to 6 mice per group.

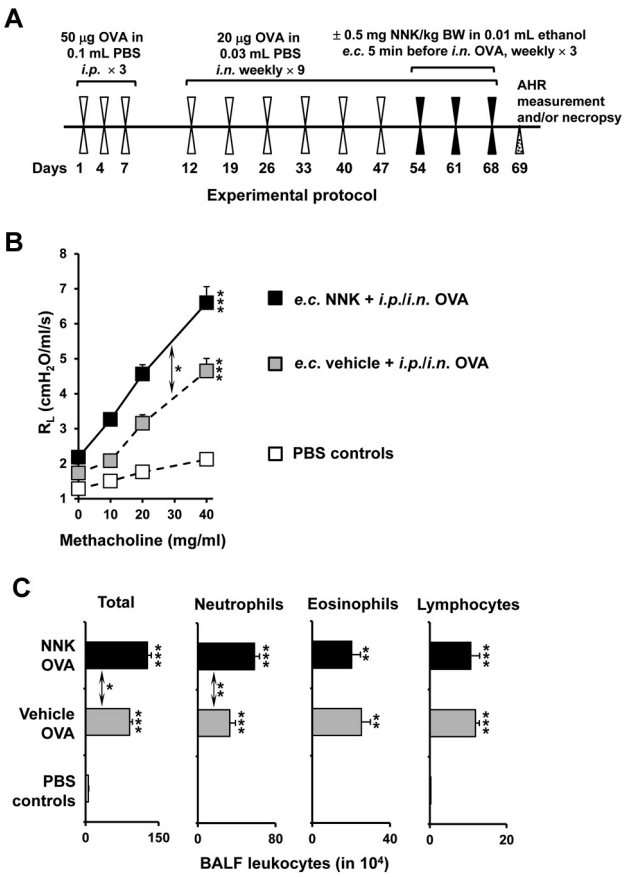


FIG E6. Influence of epicutaneous *(e.c.)* exposure to NNK on a mouse model of chronic asthma induced after OVA sensitization and challenge. **A,** Protocol: female C57BL/6 mice were sensitized with 3 intraperitoneal *(i.p.)* injections of 50 μg of OVA in 0.1 mL of PBS each, followed by 9 weekly intranasal *(i.n.)* challenges with OVA (20 μg per challenge). Some mice were subjected to epicutaneous NNK exposure on days 54, 61, and 69 five minutes before intranasal OVA challenge with 0.5 mg of NNK/kg BW in 0.01 mL of ethanol. **B,** AHR to methacholine (assessed as R_L) 24 hours after the last intranasal challenge with CRA or PBS. **C,** Numbers of individual types of leukocytes in BALF recovered from mice 24 hours after the last intranasal challenge with OVA or PBS. $^*P < .05$, $^{**}P < .01$, or $^{***}P < .001$ versus PBS-treated controls or the group indicated. N = 5 mice per group. To simplify data presentation, the PBS group depicted in the graphs includes 3 randomly selected mice from each of the intranasal PBS control groups, including PBS-treated mice with epicutaneous NNK or vehicle (ethanol) application alone. There were no statistically significant differences in the responses observed in each of these PBS-treated control groups.

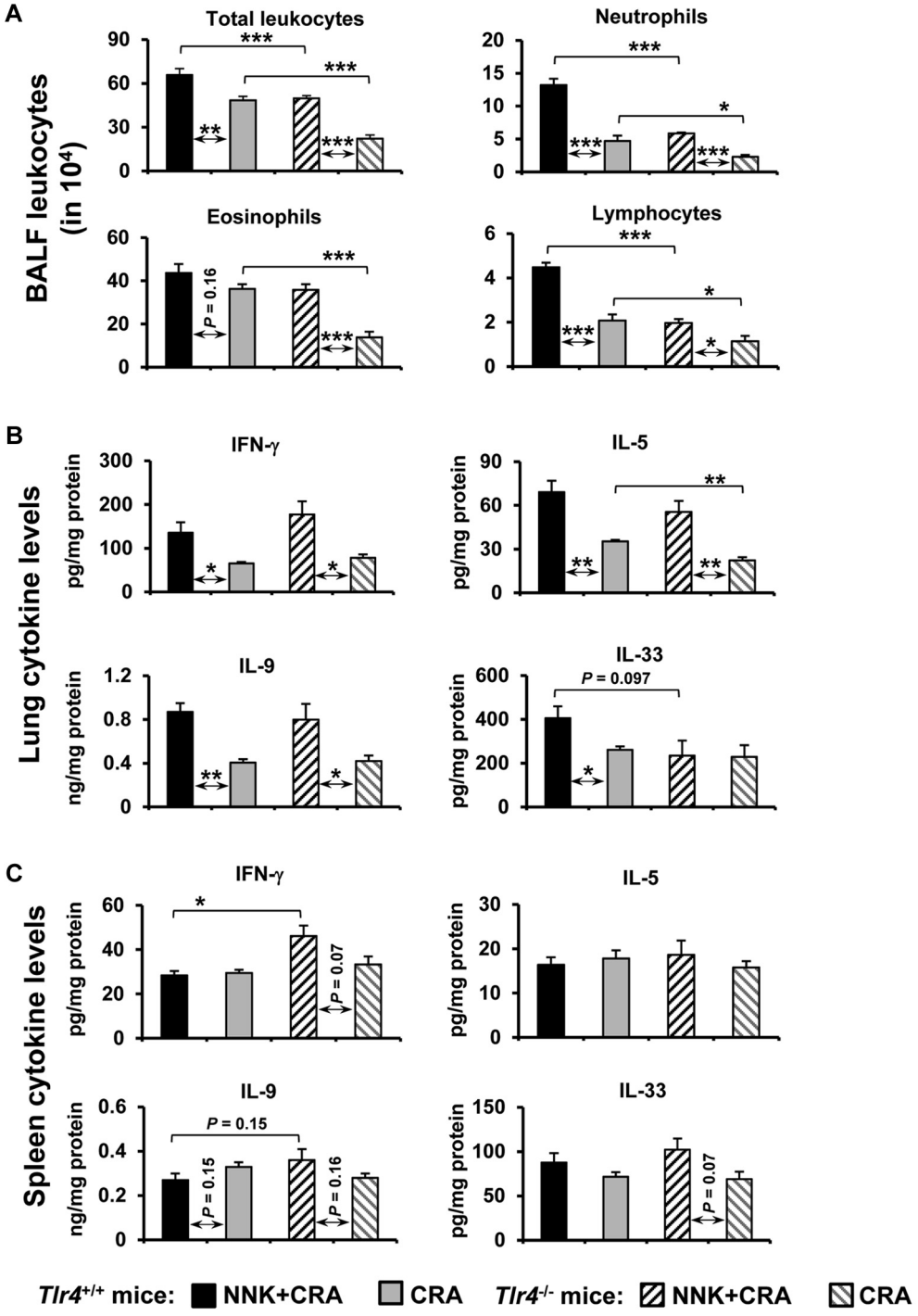


FIG E7. Influence of LPS/TLR4 signaling on development of the inflammatory features of the CRA-induced mouse model of asthma. Asthma models were elicited in female WT (*Tlr4*$^{+/+}$) or TLR4-deficient (*Tlr4*$^{-/-}$) C57BL/6 mice 10 weeks of age after intranasal CRA challenge on days 1, 2, 15, 18, and 21 with 10 μg of CRA in 0.03 mL of PBS in the presence or absence of epicutaneous *(e.c.)* exposure to NNK with 0.5 mg/kg BW in 0.01 mL of ethanol on days 1, 2, 15, 18, and 21 five minutes before intranasal CRA challenge. The profile of BALF leukocytes **(A)** and cytokine levels in the lung **(B)** and spleen **(C)** were determined 24 hours after the last CRA or PBS intranasal instillation. *$P < .05$, **$P < .01$, or ***$P < .001$ versus the group indicated. Note: $P < .05$, .01, or .001 between each of the CRA-treated groups versus the corresponding PBS control group (not presented in the figures).