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Attenuation of CNS inflammatory responses by nicotine involves $\alpha 7$ and non- $\alpha 7$ nicotinic receptors,^{1,2}

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Abstract

A considerable number of in vivo studies have demonstrated that the cholinergic system can dampen the peripheral immune response, and it is thought that the $\alpha 7$ -nicotinic acetylcholine receptor (nAChR) subtype is a key mediator of this process. The goal of the present study was to determine if nicotine modulates immunological mechanisms known to be involved in the development of experimental autoimmune encephalomyelitis (EAE), a mouse model for CNS autoimmune disease, via $\alpha 7$ -nAChRs. Here we show that nicotine exposure attenuates EAE severity and that this effect is largely abolished in nAChR $\alpha 7$ subunit knock-out mice. However, nicotine exposure partially retains the ability to reduce lymphocyte infiltration into the CNS, inhibit auto-reactive T cell proliferation and helper T cell cytokine production, down-regulate co-stimulatory protein expression on myeloid cells, and increase the differentiation and recruitment of regulatory T cells, even in the absence of $\alpha 7$ -nAChRs. Diverse effects of nicotine on effector and regulatory T cells, as well as antigen presenting cells, may be linked to differential expression patterns of nAChR subunits across these cell types. Taken together, our data show that although $\alpha 7$ -nAChRs indeed seem to play an important role in nicotine-conferred reduction of the CNS inflammatory response and protection against EAE, other nAChR subtypes also are involved in the anti-inflammatory properties of the cholinergic system.

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²Abbreviations: nAChR, nicotinic acetylcholine receptor; MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; Treg, regulatory T cell; MRI, magnetic resonance imaging

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1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are members of a diverse family of ligand-gated ion channels that serve as targets for acetylcholine and nicotine (Jensen et al., 2005). They play critical physiological roles throughout the brain and body by mediating cholinergic excitatory neurotransmission, modulating the release of neurotransmitters, and having longer-term effects on gene expression and cellular interactions (Jensen et al., 2005). Mammalian nAChR subunits are derived from a family of sixteen different genes ($\alpha 1$ – $\alpha 7$, $\alpha 9$ – $\alpha 10$, $\beta 1$ – $\beta 4$, γ , δ and ϵ) whose translation products combine as hetero- or homo-pentameric complexes to form a variety of nAChR subtypes (Jensen et al., 2005). Although many nAChR subtypes are possible in theory, differential distribution of subunits within organs limits the possibilities to an extent. In addition, there seem to be undefined rules that further limit the number of viable subunit combinations. For example, nAChR $\alpha 7$ and $\alpha 9$ subunits are known to form unique, homopentameric receptors, while other α subunits need to assemble with β subunits, in a variety of combinations, to form functional receptors (Jensen et al., 2005).

Although function of nAChRs has been studied mainly at the neuromuscular junction and in neurons, there is mounting evidence for nAChR expression and unconventional roles outside of the nervous system (Sopori, 2002, Tracey, 2009). Though somewhat inconsistent, accumulating evidence suggests that many immune cell types express one or more nAChR subunit(s) (Sato et al., 1999), including lymphocytes (Benhammou et al., 2000, De Rosa et al., 2009, De Rosa et al., 2005, Fujii et al., 2008, Sato et al., 1999), monocytes (Yoshikawa et al., 2006), macrophages (de Jonge et al., 2005, Matsunaga et al., 2001, Wang et al., 2004, Wang et al., 2003), and even endothelial cells (Wang et al., 2001). Further studies into the role of nAChRs in immune function have predominantly indicated that binding of nicotine or acetylcholine to $\alpha 7$ -nAChR leads to the suppression of inflammation (Borovikova et al., 2000, Piao et al., 2009, Wang et al., 2003), giving rise to the concept of a “cholinergic anti-inflammatory pathway”.

We have shown (Shi et al., 2009), in a study since replicated (Nizri et al., 2009), that systemic nicotine administration attenuates central nervous system (CNS) inflammation and indices of autoimmunity in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS). Both studies found that nicotine exposure inhibits the proliferation of autoreactive T cells and alters the cytokine profile of helper T cells in the periphery. In addition, numbers of CD11c⁺ dendritic (Shi et al., 2009), CD11b⁺ myeloid cells and lymphocytes (Nizri et al., 2009, Shi et al., 2009) that infiltrate the CNS were reduced in nicotine-treated mice. We also found that nicotine exposure down-regulates the expression of MHC-II, CD80 and CD86 molecules by CD11b⁺ and CD11c⁺ cells (Shi et al., 2009).

Although the involvement of $\alpha 7$ -nAChRs in CNS autoimmune disease protection has been suggested (Nizri et al., 2009), a number of critical questions remain unresolved: First, nAChR expression profiles on peripheral and CNS immune cells known to be critical for EAE development have not been comprehensively examined. Second, the extent to which $\alpha 7$ -nAChRs mediate the effect of nicotine on clinical and pathological hallmarks of EAE needs to be determined. Third, how $\alpha 7$ -nAChR deletion influences myelin-reactive T cells, regulatory T cells, as well as recruitment of myeloid cells and lymphocytes to the CNS is not clear.

In this study, we comprehensively examine nAChR subunit mRNA expression by CD4⁺ T cells, CD8⁺ T cells, monocytes/macrophages, dendritic cells, regulatory T cells (Tregs) and microglia. We reveal the partial contribution of $\alpha 7$ -nAChRs in the mediation of nicotinic

effects on the magnitude of brain inflammation, pathology and expression of EAE. Our data indicate that non- $\alpha 7$ -nAChR subtypes may also be involved in the anti-inflammatory properties of nicotine, and that the relative contributions of $\alpha 7$ - vs non- $\alpha 7$ -nAChRs may be unique to each individual immune cell type.

2. Materials and Methods

2.1 Mice

B6 (H-2^b) mice were purchased from Taconic (Germantown, NY, USA). nAChR $\alpha 7$ subunit knock-out mice were provided by M. Picciotto, Yale University, New Haven, Connecticut. FoxP3^{GFP} mice were provided by A. Rudensky, University of Washington, Seattle, Washington. CX₃CR1^{+/GFP} mice were provided by R. M. Ransohoff, Memorial Sloan-Kettering Cancer Center, New York, NY. All mutant mice were backcrossed to the B6 background for 8–11 generations. All animals were housed in pathogen-free animal facilities. Female mice used were 7 to 8 weeks of age at the experiment's inception. Experiments were reviewed, approved and conducted in accordance with institutional and NIH guidelines.

2.2 Induction of acute EAE

To induce acute EAE, mice were injected subcutaneously (s.c.) in the hind flank with 200 μ g of MOG_{35–55} peptide (single letter amino acid sequence; M-E-V-G-W-Y-R-S-P-F-S-R-V-V-H-L-Y-R-N-G-K; Bio Synthesis Inc., Lewisville, TX, USA) in complete Freund's adjuvant (CFA) (Difco, Detroit, MI, USA) containing 500 μ g of non-viable, desiccated *Mycobacterium tuberculosis*. On the day of and 2 days after immunization, the mice also were inoculated with 200 ng of pertussis toxin (List Biologic, Campbell, CA, USA) intravenously (i.v.). Mice were monitored daily for symptoms scored on an arbitrary scale of 0 to 5 with 0.5 increments: 0, no symptoms; 1, flaccid tail; 2, hindlimb weakness or abnormal gait; 3, complete hindlimb paralysis; 4, complete hindlimb paralysis with forelimb weakness or paralysis; 5, moribund or deceased.

2.3 Nicotine treatment

(–)Nicotine bitartrate was purchased from Sigma (St. Louis, MO, USA). A 100 mg/ml solution in phosphate-buffered saline (PBS) or a solution containing PBS alone was freshly prepared and loaded into Alzet® osmotic minipumps (model 1004, Durect Corporation, Cupertino, CA, USA) 24 h before pump implantation. On the day of MOG immunization, the pumps were implanted subcutaneously on the right side of the back of the mouse and continuously delivered either PBS or nicotine salt at 12 μ l/d for 28 days, and then the pumps were removed. This equated to delivery of 0.39 mg of nicotine free base per mouse per day. For a ~30 gm mouse, which is at the upper end of weight for animals used in the study, this equates to ~13 mg of nicotine free base/kg/d or ~0.54 mg of nicotine free base/kg/hr. Plasma nicotine levels in mice are ~100–200 ng/ml (~0.6–1.2 μ M) after infusion of ~2–4 mg/kg/hr of drug and ~45 ng/ml (~280 nM) after infusion at ~0.5 mg/kg/hr (Matta et al., 2007). For comparison, human smokers have peak plasma nicotine levels of 10–50 ng/ml (~60–310 nM; (Matta et al., 2007)). Thus, nicotine levels in plasma (extrapolated to be ~49 ng/ml or ~300 nM) of mice used in the studies are comparable to those in the plasma of human smokers. Some control mice received PBS via direct injections rather than through minipumps, but either delivery method produced similar results.

2.4 In vivo CNS biofluorescence and magnetic resonance imaging

For imaging of reactive oxygen species generation in brain, biofluorescence images in live mice were captured using the Xenogen IVIS200 imager (Caliper Life Sciences, Hopkinton,

MA) at several time points after injection of 27 mg/kg dihydroethidium (DHE, Molecular Probes, Eugene, OR). A region of interest (ROI) tool was used to measure fluorescence intensity. Data were collected as photons/sec/cm² using Living Image® software (Caliper Life Sciences, Hopkinton, MA).

Magnetic resonance imaging (MRI) was performed using a 7T small animal, 30-cm horizontal-bore magnet and BioSpec Avance III spectrometer (Bruker, Billerica, MA) with a 116 mm high power gradient set (600 mT/m) and a 72 mm whole-body mouse transmit/surface receive coil configuration. Axial and coronal T1-weighted (MSME; TE 10.5 ms, TR 322 ms, 0.5 mm slice thickness, matrix 256×256, field of view (FOV) 2.8 cm, eight averages, 40 coronal slices, scan time 22 minutes, and 20 axial slices, scan time 16 min) and fat-suppressed turbo spin echo T2-weighted (RARE; TE1 14.5 ms, TE2 65.5 ms, TR 4500 ms, 0.5 mm slice thickness, Matrix 256×256, FOV 2.8cm, eight averages, 40 coronal slices, scan time 28 minutes, and 20 axial slices, scan time 28 minutes) images were acquired, covering the volume of brain from the olfactory bulb/frontal lobe fissure to the cervical spinal cord. MRI data were analyzed using the MEDx3.4.3 software package (Medical Numerics, Virginia, USA) on a LINUX workstation.

2.5 T cell proliferation assays

On day 11 post-immunization, mononuclear cells were isolated from the spleen of EAE mice that were treated with nicotine or PBS. Cells were suspended in culture medium containing Dulbecco's modification of Eagle's medium (Gibco, Paisley, UK) supplemented with 1% (v/v) minimum essential medium (Gibco), 2 mM glutamine (Flow Laboratory, Irvine, CA, USA), 50 IU/ml penicillin, 50 mg/ml streptomycin, and with 10% (v/v) FCS (all from Gibco). 4×10⁵ cells in 200 µl of culture medium were placed in each well of 96-well, round-bottom microtiter plates (Nunc, Copenhagen, Denmark). Ten µl of MOG_{35–55} peptide (10 µg/ml) or Con A (5 µg/ml) (Sigma-Aldrich, St Louis, MO, USA) were then added (triplicates per condition). For the in vitro nicotine exposure experiments, cells were obtained as described, except that nicotine (final concentration of 0.1–100 µM) was also added to the culture medium in addition to MOG_{35–55} peptide. After 3 days of incubation, the cells were pulsed for 18 h with 10 µl aliquots containing 1 µCi of ³H-methylthymidine (specific activity of 42 Ci/mmol; MP Biomedicals, Irvine, CA, USA) per well. Cells were harvested onto glass fiber filters and thymidine incorporation proportional to the degree of cell proliferation was then measured. The results were expressed as counts per minute (cpm).

2.6 Spleen, lymph node and CNS cell isolation and flow cytometric analysis

Spleen and lymph node mononuclear cell suspensions were collected from PBS- or nicotine-treated mice on day 11 (i.e., at the peak of the EAE response). Single cell suspensions were prepared and stained for one or more of the following antigens (targeted by the indicated antibody fluorescently tagged with either FITC, PE, Allophycocyanin, PE-Cy5 or PE-Cy7): CD25 (PC61.5), CD3 (17A2), CD4 (GK1.5), CD8 (53–6.7), CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD45 (104), CD80 (16–10A1), CD86 (GL1), and MHC class II (M5/114.15). Intracellular Foxp3 (FJK-16s) staining was performed according to the manufacturer's protocol (eBioscience, San Diego, CA, USA). Appropriate isotype controls were always included. All samples were analyzed on a FACSAria™ using Diva. The absolute number of a particular cell subset was calculated based on the percentage of cells stained for the appropriate markers determined by FACSAria flow cytometry and the number of mononuclear cells per mouse spleen defined based on hemocytometer counts.

For CNS cell isolates, at day 11 after EAE induction, mice were sacrificed and perfused with PBS delivered transcardially to eliminate contaminating blood cells in the CNS. CNS

mononuclear cells were then isolated from five to six mice based on their characteristic sedimentation features on Percoll density gradients (30% ~70%) and stained for cell surface markers as for splenocytes. Antibodies were directly labeled and analyzed as done for splenocytes. Absolute numbers and percentages of particular, CNS mononuclear cell subsets were determined as described above and previously (Bai et al., 2004).

2.7 Isolation of Treg cells

We isolated Treg cells from the lymph nodes and CNS of mice expressing a green fluorescent protein (GFP) “knocked” into a FoxP3 allele. The GFP reporter gene allows us to conveniently isolate this population and perform phenotype and functional experiments, as described previously (Fontenot et al., 2005). Cell purity reaches ~97% after sorting.

2.8 Isolation of microglial cells

We used mice in which the CX3CR1 gene was replaced with a cDNA encoding GFP, such that CX3CR1^{+/−} (CX3CR1^{+/GFP}) mice express the GFP reporter in cells that retain their receptor function, whereas CX3CR1^{−/−} (CX3CR1^{GFP/GFP}) cells are also labeled and lack CX3CR1 expression (Cardona et al., 2006a, Cardona et al., 2006b). Since CX3CR1-GFP reporter mice can exclusively identify microglia *in vivo*, the CX3CR1^{+/GFP} mice permitted us to isolate and purify these cells *ex vivo* (Cardona et al., 2006a, Cardona et al., 2006b). Cell purity reaches >97% after sorting.

2.9 Cytokine quantification

Single cell suspensions of splenic mononuclear cells were incubated at 37°C for 3 days in round-bottom plates (2 × 10⁶ cells/well) with or without antigens (MOG 10 µg/ml, PLP 10 µg/ml or Con A 2.5 µg/ml) and then stimulated with phorbol myristate acetate (PMA 20 ng/ml)/ionomycin (1 µg/ml)/brefeldin A (5 µg/ml) for 5 h at 37°C. After harvesting, cells were stained for surface markers with fluorochrome-conjugated monoclonal antibodies targeting CD3, CD4 and/or CD8 as described above and/or for intracellular cytokines using anti-IFN-γ, anti-IL-4, anti-IL-10 or anti-IL-17 monoclonal antibodies conjugated with Alexa 647 after fixation and permeabilization using Cytofix/Cytoperm kit (BD Bioscience). All samples were analyzed on a FACS Aria™ using Diva. For assessment of effects of treatments on cytokine production, supernatants were collected 3 days after *in vitro* boosting. IFN-γ, IL-10, IL-2, IL-4, IL-17 and TGF-β were measured using optEIA kits (PharMingen and eBioscience).

2.10 mRNA purification and reverse-transcription PCR

mRNA was purified from fresh acutely-isolated cells (~1.5 × 10⁶ cells per sample) by using the µMACS mRNA isolation kit (Miltenyi Biotec), as per the supplied protocol. Reverse transcription was performed with the SuperScript III First Strand cDNA Synthesis kit (Invitrogen, USA) by following the supplied protocol. Oligo-dT sequences were used to prime the reverse transcriptase. PCR was then performed following established protocols, using a variety of primers that are specific to each target mRNA (Table I). Primer pairs were designed with the use of PubMed’s “Primer-blast” tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). For each pair, forward and reverse primers were specific to different exons, so that potential DNA contamination could be ruled out. PCR was performed using the RedTaq PCR kit (Sigma, USA), according to the supplied protocol. Primers were always tested with total brain cDNA (positive control), and the optimal annealing temperature (T_m) was determined empirically (with the use of a Temperature gradient thermocycler), while maintaining high stringency conditions. A negative control (no cDNA template), was included in every set of reactions. PCR conditions were the following: 95 °C for 5 minutes, followed by 35–40 cycles of 95 °C for 1

minute, 55–58 °C for 1 minute and 72 °C for 1.5 minutes, and a final extension step of 72 °C for 10 minutes. PCR products for each primer pair from positive controls, microglial and CD4⁺ T cell samples were sequenced in order to ensure the accuracy of our results.

2.11 Statistical analysis

Differences between groups were evaluated by performing ANOVA. Fisher's exact test and Mann-Whitney's *U* test were applied to analyze disease incidence and severity, respectively, of symptomatic manifestations.

3. Results

3.1 Protective effects of nicotine against EAE are reversed in nAChR $\alpha 7$ subunit knock-out mice

The $\alpha 7$ -nAChR subtype has been considered to be the principal constituent of the cholinergic anti-inflammatory pathway (Wang et al., 2003). To determine the extent to which the $\alpha 7$ -nAChR subtype mediates the protective effects of nicotine in EAE, wild type ($\alpha 7^{+/+}$; WT) or heterozygous ($\alpha 7^{+/-}$)/homozygous ($\alpha 7^{-/-}$; KO) nAChR $\alpha 7$ subunit knock-out mice were immunized with MOG/CFA and treated with PBS or nicotine. Immunization of PBS-treated $\alpha 7^{-/-}$ mice produced EAE at a similar magnitude to that produced in PBS-treated wild type $\alpha 7^{+/+}$ mice, indicating that nAChR $\alpha 7$ subunit deletion by itself does not alter disease onset or progression (Fig. 1A). We previously demonstrated that seven days of nicotine treatment started seven days prior to, on the day or, or seven days after MOG_{35–55} peptide immunization delayed the onset of EAE and reduced the severity of disease in wild type mice (Shi et al., 2009). In the current studies, nicotine delivery via minipump started as before on the day of MOG_{35–55} peptide immunization (induction of EAE) but continued for 28 days. In support of our original findings (Shi et al., 2009), since verified (Nizri et al., 2009), and indicating that such treatment for 7 or 28 days have the same effect, nicotine treatment delayed the onset of EAE and reduced the severity of disease in $\alpha 7^{+/+}$ mice. Nicotine treatment-induced protection against EAE development was partially reduced in heterozygous $\alpha 7^{+/-}$ mice (Suppl. Fig. 1) and completely absent in homozygous $\alpha 7^{-/-}$ mice (Fig. 1A; see also Nizri et al., 2009).

CNS inflammation and demyelination are pathological hallmarks of EAE. We thus quantitatively assessed inflammatory changes with the use of bioluminescence imaging of DHE-labeled reactive oxygen species, and brain lesions were qualitatively observed using high field MRI. Inflammation was equally pronounced in PBS-treated $\alpha 7^{+/+}$ or $\alpha 7^{-/-}$ EAE mice (Fig. 1B). $\alpha 7^{+/+}$ mice that received nicotine had significantly less inflammation in the CNS ($p < 0.01$ vs PBS-treated $\alpha 7^{+/+}$ mice), whereas the drug had a moderate, but not statistically significant, effect in $\alpha 7^{-/-}$ mice ($p = 0.067$ for nicotine-treated vs PBS-treated $\alpha 7^{-/-}$ mice). Similarly, nicotine exposure diminished EAE-induced brain lesions, as revealed by MRI in $\alpha 7^{+/+}$ mice ($p < 0.01$), but had only a partial effect in $\alpha 7^{-/-}$ mice ($p = 0.051$; Fig. 1C). Taken together, these data confirm previous work showing that the $\alpha 7$ -nAChR subtype plays a significant role in the overall protective and anti-inflammatory effects of nicotine in EAE (Nizri et al., 2009). However, this raises the question as to whether nicotinic effects on each individual immune cell type implicated in EAE (Shi et al., 2009) are mediated by the $\alpha 7$ -nAChR subtype.

3.2 Nicotine-induced alterations of lymphocyte subpopulations, proliferation and Th cell cytokine profile are partially reversed in nAChR $\alpha 7$ subunit knock-out mice

We have recently shown that nicotine treatment reduces the numbers and percentage of various lymphocyte subpopulations, both in the periphery and CNS of EAE mice (Shi et al., 2009). Thus, we first defined if the $\alpha 7$ -nAChR subtype is involved in the nicotinic

modulation of lymphocyte subpopulations by quantifying the percentage and numbers of various lymphocyte cell populations in the spleens and CNS of $\alpha 7^{+/+}$ or $\alpha 7^{-/-}$ EAE mice treated either with PBS or nicotine. In the periphery (Fig. 2A, B; $n = 6$ per group), nicotine treatment lowered the proportions and numbers of $CD3^+$, $CD4^+$ and $CD8^+$ T cells, as well as $CD3^-/CD19^+$ B cells, in the spleens of $\alpha 7^{+/+}$ EAE mice ($p < 0.05$). nAChR $\alpha 7$ subunit deletion had no effect on these cell types in PBS-treated animals. Interestingly, the amounts of $CD3^+$, $CD4^+$ and $CD8^+$ T cells in the spleens of nicotine-treated $\alpha 7^{-/-}$ EAE mice were not statistically different than in either the nicotine-treated wild type EAE mice or the PBS-treated $\alpha 7^{-/-}$ EAE mice ($p = 0.058$, 0.073 and 0.062 , respectively; Fig. 2A, B), suggesting that nicotine still had a partial effect on peripheral T cell subpopulations in $\alpha 7^{-/-}$ mice. On the other hand, nicotine retained the ability to reduce the number of B cells ($CD3^-/CD19^+$) in the spleens of $\alpha 7^{-/-}$ mice ($p < 0.05$).

We next defined the lymphocyte subpopulations in the CNS of EAE mice (Fig. 2C, D). Due to the amount of cell loss during the cell purification process and much smaller lymphocyte sample sizes in the CNS, compared to peripheral samples obtained from the spleen, brains of at least 5–6 animals per group were pooled prior to FACS analysis. As expected, there were fewer $CD3^+$, $CD4^+$ and $CD8^+$ T cells and $CD19^+$ B cells in the CNS of nicotine-treated $\alpha 7^{+/+}$ mice compared to wild-type mice that received PBS ($p < 0.05$). On the other hand, the effect of nicotine exposure on the amount of lymphocytes within the CNS was only partially reversed in $\alpha 7^{-/-}$ mice ($p < 0.05$ compared to either nicotine-treated $\alpha 7^{+/+}$ or PBS-treated $\alpha 7^{-/-}$). nAChR $\alpha 7$ subunit deletion had no effect on the amounts of these cells after PBS treatment (Fig 2C, D). Taken together, these data suggest that non- $\alpha 7$ -nAChRs are also involved in nicotinic modulation of lymphocyte recruitment to the spleen and CNS.

Nicotine exposure inhibits autoreactive T cell expansion (Nizri et al., 2009, Shi et al., 2009). To determine if this effect is dependent upon the $\alpha 7$ -nAChR subtype, we obtained mononuclear cells from the spleens of $\alpha 7^{+/+}$ or $\alpha 7^{-/-}$ EAE mice treated either with PBS or nicotine. These cells were then cultured and stimulated with either MOG_{35–55} or Con A, and proliferation was then assessed by measuring the incorporation of 3H -methylthymidine into the cells (Fig. 3A; $n = 6–8$ per group). Compared to baseline responses in tissue culture medium, both methods of stimulation induced considerable proliferation in cells obtained from PBS-treated $\alpha 7^{+/+}$ or $\alpha 7^{-/-}$ animals. No differences were observed between $\alpha 7^{+/+}$ and $\alpha 7^{-/-}$ animals that received PBS. In support of previous results, nicotine prevented MOG-, but not ConA-, induced T cell proliferation in $\alpha 7^{+/+}$ mice ($p < 0.05$). The fact that nicotine does not affect ConA-induced proliferation suggests that antigen-specific signal strength may determine the responsiveness of lymphocytes to nicotine. Although the proliferation of cells taken from $\alpha 7^{-/-}$ mice was higher than the $\alpha 7^{+/+}$ group following nicotine treatment, we observed a tendency towards lower proliferation of nicotine-treated $\alpha 7^{-/-}$ T cells as compared to their PBS-treated counterparts ($p = 0.059$).

In a separate set of experiments (Fig. 3B), similar conditions were used except that cells taken from $\alpha 7^{+/+}$ or $\alpha 7^{-/-}$ animals that received either PBS or nicotine were further incubated with nicotine at various concentrations (0.1–100 μM) in addition to MOG_{35–55}. As expected, proliferation of cells obtained from nicotine-treated $\alpha 7^{+/+}$ mice was severely blunted at all concentrations of nicotine applied in vitro relative to proliferation of cells taken from PBS-treated $\alpha 7^{+/+}$ mice ($p < 0.01$). In vitro nicotine exposure showed further, concentration-dependent reduction of T cell proliferation whether cells were taken from wild type mice that were treated with nicotine or PBS ($p < 0.05$ comparing data for 0.1 μM nicotine to results for 10 or 100 μM nicotine in vitro). Surprisingly, but similarly, nicotine treatment in vitro also inhibited proliferation of cultured T cells taken from either PBS- or nicotine-treated $\alpha 7^{-/-}$ mice in a concentration-dependent manner ($p < 0.05$ comparing results at 0.1 μM to 1 or 10 μM), implicating roles for non- $\alpha 7$ -nAChR in the in vitro effects of

nicotine. Also of note, regardless of nicotine concentration applied *in vitro*, cells from $\alpha 7^{-/-}$ mice that received nicotine *in vivo* had reduced T cell proliferation compared to their PBS-treated counterparts ($p < 0.05$). Collectively, these findings provide further evidence that nicotine exposure inhibits T cell proliferation via effects that are blunted but not eliminated in nAChR $\alpha 7$ knock out animals and therefore are likely, mediated at least in part via non- $\alpha 7$ -nAChRs.

In light of our previous finding that nicotine treatment induces a shift from Th1 towards Th2 cytokine secretion (Shi et al., 2009), we assessed whether this effect is achieved via the $\alpha 7$ -nAChR subtype. As expected, nicotine exposure in wild type mice relative to PBS-treated control $\alpha 7^{+/+}$ animals substantially inhibited IFN- γ , IL-2 and IL-17 secretion from splenic mononuclear cells, although the effect on IL-17 did not achieve statistical significance (Table II; $n = 6-8$ per group). On the other hand, nicotine treatment induced statistically significant increases in the release of IL-10 and TGF- β relative to PBS exposure in wild type mice. nAChR $\alpha 7$ subunit knock out alone had no effect on cytokine secretion ($p > 0.05$ comparing PBS-treated wild type and $\alpha 7^{-/-}$ mice). In all cases but for TGF- β , nicotinic effects on cytokines were observed even in the absence of $\alpha 7$ -nAChR, albeit to a slightly lesser extent ($p < 0.05$ in $\alpha 7^{-/-}$ nicotine-treated compared to PBS-treated animals for IFN- γ and IL-10; trend for IL-2 and IL-17), suggesting once again that non- $\alpha 7$ -nAChRs may additionally be involved in the anti-inflammatory properties of nicotine.

Nicotine exposure also increases Treg cell differentiation and recruitment to the CNS during EAE (Shi et al., 2009). Compared with PBS treatment, nicotine treatment in $\alpha 7^{+/+}$ EAE mice, increased ($p < 0.05$) the amount of Treg cells found in lymph nodes and the CNS (Fig. 4A–D), confirming our previous findings. Under control conditions of PBS treatment, nAChR $\alpha 7$ subunit knock out animals do not differ from wild type animals in Treg cell differentiation and CNS recruitment. Increased proportions of Treg cells in the periphery and CNS are seen upon nicotine treatment of $\alpha 7^{-/-}$ mice relative to PBS-treated controls ($p < 0.05$), but the magnitude of the nicotine effect is blunted in $\alpha 7^{-/-}$ relative to $\alpha 7^{+/+}$ mice ($p < 0.05$). These results imply that nicotine modulates the differentiation and recruitment of Treg cells to the CNS primarily via the $\alpha 7$ -nAChR subtype, but that other nAChR subtypes also may take part in this process.

3.3 Changes in peripheral and CNS myeloid cell populations

It was recently shown that nicotine treatment in EAE-induced $\alpha 7^{+/+}$ mice considerably diminishes the numbers of CD11b $^{+}$ (Nizri et al., 2009, Shi et al., 2009) and CD11c $^{+}$ (Shi et al., 2009) cells in the CNS. Moreover, there were significant reductions in the proportions and absolute numbers of CD11b $^{+}$ and CD11c $^{+}$ cells that also expressed high levels of MHC-II, CD80 and CD86, both in the periphery and the CNS (Shi et al., 2009). Importantly, the magnitude of reduction in the expression of these co-stimulatory molecules in myeloid cells harvested from animals exposed to nicotine was much greater in the CNS than in the periphery (Shi et al., 2009). We thus assessed whether nicotine exerts these effects on myeloid cells by targeting the $\alpha 7$ -nAChR subtype.

Consistent with our previous report (Shi et al., 2009), nicotine treatment significantly inhibited the recruitment of CD11b $^{+}$ and CD11c $^{+}$ cells into the CNS of wild type mice ($p < 0.05$ compared to PBS-treated animals; Fig. 5A, D). There was no difference in cell numbers between nAChR $\alpha 7$ subunit knock out and wild type animals in the absence of nicotine exposure. Nicotine treatment also attenuated CNS recruitment of CD11b $^{+}$ and CD11c $^{+}$ cells in $\alpha 7^{-/-}$ mice relative to PBS-treated $\alpha 7^{-/-}$ mice, but to a lesser extent than seen for nicotine-treated $\alpha 7^{+/+}$ mice ($p < 0.05$ in both cases; Fig. 5A, D). Furthermore, there was a decline in the expression of MHC-II, CD80 and CD86 by CD11b $^{+}$ cells (Fig. 5B, C; $p < 0.05$), but not CD11c $^{+}$ cells (Fig. 5E, F), taken from the spleens or CNS of nicotine-

treated compared to PBS-treated $\alpha 7^{+/+}$ EAE mice. Interestingly, nAChR $\alpha 7$ subunit deletion had moderate effects on the ability of nicotine exposure to reduce MHC-II and CD80 expression in both splenic and CNS CD11b⁺ cells. Conversely, nicotine treatment did not affect CD86 expression in CD11b⁺ cells taken from $\alpha 7^{-/-}$ mice. Taken together, these data suggest that nicotine modulates myeloid cell infiltration into the CNS, and the expression of co-stimulatory molecules by CD11b⁺ cells, via multiple nAChR subtypes, including $\alpha 7$ -nAChRs.

3.4 nAChR mRNA expression

To this point, our findings demonstrate that other nAChR subtypes, in addition to $\alpha 7$ -nAChRs, may be involved in the mediation of the anti-inflammatory properties of nicotine in EAE. We therefore defined whether there is differential expression of nAChR subunits as mRNA in acutely isolated peripheral T cells (CD4⁺ and CD8⁺ subsets), monocytes/macrophages (CD11b⁺/CD45⁺), dendritic cells (CD11c⁺/CD45⁺), regulatory T cells and CNS microglia taken from $\alpha 7^{+/+}$ and $\alpha 7^{-/-}$, naïve or EAE mice by using RT-PCR (Fig. 6). To this end, we obtained highly purified cells (>95% pure; Suppl. Fig. 2) by FACS 7 days post-MOG immunization for mRNA extraction. CD4⁺ cells (n = 4), CD8⁺ cells (n = 4), Treg cells (n = 8), monocytes/macrophages, and dendritic cells were obtained from the spleens of the indicated numbers of animals, whereas microglia (n = 3) were purified from the CNS.

Mouse brain cDNA (B) served as a positive control and indeed showed expression of all nAChR subunit genes as message, also validating protocols. Microglial cells (M) taken from naïve WT mice expressed all nAChR subunits studied. We found that naïve WT CD4⁺ T cells express nAChR $\alpha 5$, $\alpha 7$ (barely detectable even after 40 cycles), $\alpha 9$ and $\beta 2$ subunits, whereas other subunit mRNAs were either absent or below the threshold of detection. Interestingly, mRNA levels for nAChR $\alpha 6$, $\alpha 7$ and $\beta 4$ subunits increased or became detectable in CD4⁺ T cells taken from MOG-immunized WT mice. Conversely, CD8⁺ T cells taken from naïve WT mice expressed nAChR $\alpha 4$, $\alpha 5$, $\alpha 7$ (barely detected), $\alpha 9$, $\beta 2$ and $\beta 4$ subunits. nAChR $\alpha 7$ subunit mRNA was more readily detectable in CD8⁺ T cells following MOG-immunization. In monocytes/macrophages (CD11b⁺) and dendritic cells (CD11c⁺), mRNA was detected for nAChR $\alpha 4$, $\alpha 5$, $\alpha 7$, $\alpha 9$, $\beta 2$ and $\beta 4$ subunits. MOG-immunization induced a slight decrease in nAChR $\alpha 7$ subunit mRNA in both monocytes/macrophages and dendritic cells and caused expression of the nAChR $\alpha 5$ subunit gene to no longer be detectable in dendritic cells, in which there seems to be a slight increase in nAChR $\alpha 4$ subunit expression. Interestingly, we failed to detect the presence of any nAChR subunit mRNA in Treg cells taken from WT naïve mice, although further verification from Treg cells isolated from EAE mice might consolidate this finding.

We also compared mRNA expression in $\alpha 7^{+/+}$ vs $\alpha 7^{-/-}$ mice. No expression of the nAChR $\alpha 7$ subunit gene was found, as expected, in $\alpha 7^{-/-}$ mice. We did not find any major differences between wild type and knock out animals in mRNA levels for other nAChR subunits except that nAChR $\alpha 4$ subunit mRNA was not detected in CD8⁺ T cells from $\alpha 7^{-/-}$ mice. nAChR $\beta 2$ subunit mRNA levels also may be lower in $\alpha 7^{-/-}$ mouse CD11b⁺ cells. Effects of MOG immunization on nAChR subunit gene expression in cells derived from $\alpha 7^{-/-}$ mice are not notably different from effects in cells from wild type animals. The fact that all cell types studied, with the exception of naïve Treg cells, have mRNAs for nAChR subunits other than the $\alpha 7$ subunit is consistent with our data showing that nicotine modulates inflammation via multiple nAChR subtypes.

4. Discussion

The principal findings in this study are that although $\alpha 7$ -nAChR deletion prevents nicotine from protecting against clinical manifestations in EAE mice, many parameters relating to

inflammatory processes involved in an autoimmune response and affected by nicotine exposure are only partially attenuated in $\alpha 7^{-/-}$ mice. These include changes in lymphocyte infiltration into the CNS, proliferation of auto-reactive T cells, Th cell cytokine profiles, the differentiation and recruitment of Treg cells to the CNS, and the expression of co-stimulatory molecules on myeloid cells. Of note, all of the immune cell types included in this study, with the exception of Treg cells, express mRNA for multiple nAChR subunits. Thus, whereas these findings support a previously-reported role in nicotine-induced protection in EAE (Shi et al., 2009) for $\alpha 7$ -nAChR (Nizri et al., 2009), other subunits might combine to form nAChR subtypes that also contribute to nicotine's anti-inflammatory effects and to the roles of nicotinic cholinergic signaling in autoimmunity. Moreover, $\alpha 7$ -nAChR involvement in nicotinic modulation of Treg cell differentiation and function might occur indirectly through the expression of these receptors by other cell types, such as microglia or peripheral monocytes/macrophages and dendritic cells.

Initiation of MS and EAE clearly requires the activation of T cells in peripheral lymphoid organs. However, disease initiation also involves the infiltration of myelin-reactive T cells into the CNS, which are reactivated centrally by local antigen-presenting cells (APCs). Once reactivated, T cells couple with other cellular and soluble components of the immune system to orchestrate the induction of CNS pathology in EAE (Ponomarev et al., 2007). Nicotine has previously been shown to attenuate EAE severity and to reduce the recruitment of $CD4^{+}$ T cells to the CNS (Shi et al., 2009), a process that was suggested to be mediated by the $\alpha 7$ -nAChR subtype (Nizri et al., 2009). In this study, we confirm that $\alpha 7$ -nAChRs play an important role in the protection conferred by nicotine against EAE. However, the fact that the protective effects of nicotine against EAE symptoms and pathology are completely reversed in nAChR $\alpha 7$ subunit knock-out mice does not automatically imply that nicotinic effects on individual components of the immune response are also solely mediated by this receptor subtype. Indeed, we discovered that nicotinic effects on the recruitment of leukocytes to the CNS, on the proliferation of lymphocytes, and on Th1 cytokine production were only partially reversed in $\alpha 7^{-/-}$ mice.

As to what nAChR subtypes other than $\alpha 7$ -nAChR could mediate nicotine's effects, and via which cell types, our PCR analyses revealed that $CD4^{+}$ and $CD8^{+}$ T cells from the spleens of naïve (non-EAE), wild type mice have mRNA for nAChR $\alpha 5$, $\alpha 7$, $\alpha 9$ and $\beta 2$ subunits, whereas $CD8^{+}$ T cells additionally express nAChR $\alpha 4$ and $\beta 4$ subunit genes as mRNA. Of note, nAChR $\alpha 7$ subunit mRNA was barely detectable in both T cell subsets. $CD11b^{+}$ monocytes/macrophages have mRNA for nAChR $\alpha 5$, $\alpha 7$, $\alpha 9$, $\beta 2$ and $\beta 4$ subunits, and dendritic cells additionally express the nAChR $\alpha 4$ subunit gene. Surprisingly, we were unable to detect mRNA for any nAChR subunit in regulatory T cells. Conversely, microglial cells expressed mRNA for each nAChR subunit assessed in this study. Thus, in addition to $\alpha 7$ -, $\alpha 9$ -, $\alpha 3^{*}$ -, $\alpha 4^{*}$ -, and $\alpha 6^{*}$ -nAChR are candidate receptor subtypes also found in the CNS that could contribute to nicotine's effects, but it also is possible that other, novel nAChR subtypes with different subunit compositions could be active in immune and inflammatory responses. Microglia, T cells, macrophage/monocytes, and dendritic cells all are candidate hosts for mediation of nicotine effects. Perhaps of importance, deletion of nAChR $\alpha 7$ subunit seems to affect nAChR $\alpha 4$ subunit in $CD8^{+}$ T cells, but produces no other differences in nAChR subunit gene expression, placing a limit on roles played by gene expression compensatory mechanisms in the loss of nicotine's protection against EAE in $\alpha 7^{-/-}$ mice. Overall, our data demonstrate that most of the immune cells included in our study express substrates in addition to nAChR $\alpha 7$ subunits that can account for non- $\alpha 7$ -nAChR-mediated effects of nicotine in the EAE model or CNS autoimmunity and inflammation.

Interestingly, immunization of wild type mice with MOG_{35–55} induced apparent increases in expression of the nAChR $\alpha 7$ subunit gene in both T cell subsets but decrease in macrophage/monocyte and dendritic cells. MOG Immunization also induced expression of nAChR $\alpha 6$ and $\beta 4$ subunit genes in CD4⁺ T cells and abolished expression of nAChR $\alpha 5$ subunit genes in dendritic cells. This suggests that environmental influences – in this case, MOG immunization and perhaps its sequelae – can influence nAChR subunit gene expression patterns, just as occurs during development of the immune system (Kuo et al., 2002).

Our data are consistent with previous studies showing that $\alpha 7$ -nAChR-like radioligand binding sites are present in total lymphocyte populations (De Rosa et al., 2009, De Rosa et al., 2005, Sato et al., 1999, Yoshikawa et al., 2006), immortalized T cell lines (Oloris et al., 2009, Razani-Boroujerdi et al., 2007) and primary cultures of CD4⁺ T cells (Nizri et al., 2009). Our finding that nAChR $\alpha 7$ subunit message levels in T cells increase after MOG immunization suggests that levels of receptor-like binding sites also might increase in response to such a challenge and perhaps upon growth in primary cell culture or after immortalization (Oloris et al., 2009). Further work is needed to define the dynamics of nAChR expression in immune cells, as their mediation of nicotinic effects also could vary depending on conditions and models used.

In our previous study, it was shown that nicotine treatment increases the number of Treg cells in the periphery and in the CNS of wild-type EAE mice (Shi et al., 2009). Interestingly, we now demonstrate for the first time that these effects of nicotine are at least partially attenuated in nAChR $\alpha 7$ subunit knock-out mice. In addition, we did not detect nAChR subunit mRNA in peripheral Treg cells. Our data thus suggest that nicotine modulates Treg cells via an indirect mechanism involving other cell types that express nAChRs, and that such action occurs through multiple nAChR subtypes. More work is needed to define the precise mechanism(s) and cell type(s) that mediate the nicotinic modulation of Treg cell recruitment to the CNS.

APCs including monocytes, macrophages and microglia can influence T cell recruitment and function by releasing cytokines and chemokines (Columba-Cabezas et al., 2003, O'Brien et al., 2008). Once within the CNS, myelin-reactive T cells need to be re-activated by local APCs in order to initiate EAE pathology. Circulating monocytes that infiltrate the CNS in the early stages of EAE are considered to be one of the key APC types involved in this process (King et al., 2009). Furthermore, microglia are able to up-regulate expression of co-stimulatory proteins and can thus also serve as APCs (Ponomarev et al., 2005). In this study, we found that nicotine reduces CD11b⁺ and CD11c⁺ cell infiltration and inhibits the expression of co-stimulatory proteins by CNS CD11b⁺ cells (microglia and infiltrated monocytes/macrophages). Both of these effects were partially dependent on the presence of the $\alpha 7$ -nAChR subtype. We therefore assessed nAChR subunit gene expression as mRNA in microglia taken from naïve adult mice, and we found that microglia constitutively have mRNA for all nAChR subunits assessed in this study. Our findings add to previous reports showing that cultured neonatal microglia express nAChR $\alpha 7$ subunit genes as mRNA and protein (De Simone et al., 2005, Shytle et al., 2004, Suzuki et al., 2006). The presence of various nAChR subunit mRNAs in microglia supports the notion that nicotine may modulate microglial function by targeting multiple nAChR subtypes. More importantly, the fact that these cells express nAChR $\alpha 7$ subunits, combined with our findings that nicotine may indirectly mediate Treg differentiation and recruitment, suggests that microglia and infiltrating macrophages may be important targets of nicotine within the CNS, at least for the EAE model of MS. However, it remains to be determined whether nicotine modulates immune function by acting directly on receptors expressed in these cells or indirectly through other cell types.

The fact that differential nAChR subunit gene expression profiles were observed between various immune cells suggests possible and novel therapeutic avenues for the treatment of autoimmune disorders. Each nAChR subtype has unique pharmacological and biophysical properties. Accordingly, function of cells involved in inflammatory/autoimmune responses might be differentially modulated by targeting the nAChR subtypes they express. This underscores the importance of defining the functional (in addition to the gene expression profile as done here) nAChR phenotype of specific cell types in the neuroimmune axis as well as the roles played by those receptor and cell types. nAChR subtype-selective ligands could be used to specifically target a given cell type to have rather selective effects on neuroimmune function. As a practical example, our current data suggest that an $\alpha 7$ -nAChR-selective agonist might inhibit the development of EAE without completely altering autoimmune/inflammatory cell reactivity. This could mean that such drugs would have a lower, adverse side-effect liability. Similarly, inhibition of $\alpha 7$ -nAChR function could be leveraged to combat undesired responses. In any case, nAChR subtype-selective therapies could potentially be used alone as a novel treatment or in combination with currently existing treatments to increase overall therapeutic efficacy.

In summary, although we confirm that $\alpha 7$ -nAChRs play an important role in nicotine-dependent protection against EAE, we also demonstrate that other nAChRs must be involved in this process, because many features of inflammatory and autoimmune responses affected by nicotine exposure are not entirely lost in nAChR $\alpha 7$ subunit knockout mice. Moreover, we provide evidence that CD4⁺ and CD8⁺ T cells, peripheral monocytic and dendritic cells, as well as CNS microglia, all of which are important mediators of EAE etiology, express multiple nAChR subunit genes as mRNA. These data demonstrate that cholinergic modulation of inflammation is not solely dependent on the $\alpha 7$ -nAChR subtype and is thus more elaborate than previously thought, likely involving several nAChR subtypes that could be expressed by a variety of cell types. The precise role(s) of other nAChR subtypes in the regulation of inflammation and autoimmunity warrants further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Clinical and pathological features of EAE in nicotine-treated, nAChR $\alpha 7$ subunit knock-out mice

(a) Wild type ($\alpha 7^{+/+}$) or $\alpha 7$ nAChR knock-out ($\alpha 7^{-/-}$) mice were immunized with MOG/CFA. Mice received nicotine (Nic) or PBS for 28 days upon EAE induction ($n = 6-8$ per group). Differences in clinical scores (mean $\pm$ SEM; ordinate) are significant ($p < 0.05$) at day 10 post-immunization between the $\alpha 7^{+/+}$ Nic group ($\bullet$) and the other groups, but not ($p > 0.05$) for comparisons across $\alpha 7^{+/+}$ PBS ($\blacktriangle$), $\alpha 7^{-/-}$ PBS ($\blacktriangledown$), and $\alpha 7^{-/-}$ Nic ($\circ$) groups. Cumulative disease scores are also depicted. (b) Visualization and quantification of brain inflammation by in vivo bioluminescence imaging (billions of photons/sec/cm² $\pm$ SEM; ordinate) at day 14 post-immunization and PBS/nicotine treatment for $\alpha 7^{+/+}$ PBS (solid bars), $\alpha 7^{+/+}$ Nic (open bars), $\alpha 7^{-/-}$ PBS (gray bars), or $\alpha 7^{-/-}$ Nic (cross-hatched bars) animals. (c) T2 weighted periventricular images were obtained with a 7T MRI scanner 14 days after immunization plus nicotine/control treatment. Arrows indicate focal lesions located around the lateral ventricles and increased signal intensity. Pathology and imaging experiments were conducted in groups of 6 mice. Bar graphs show quantified data (lesion volume $\pm$ SEM in mm³; ordinate) for $\alpha 7^{+/+}$ PBS (solid bars), $\alpha 7^{+/+}$ Nic (open bars), $\alpha 7^{-/-}$ PBS (gray bars), or $\alpha 7^{-/-}$ Nic (cross-hatched bars) animals (* = $p < 0.05$, ** = $p < 0.01$). Nicotine is unable to suppress EAE severity and has moderate effects on overall CNS inflammation in $\alpha 7^{-/-}$ mice.

Fig. 2. Altered recruitment of lymphocytes to the CNS of $\alpha 7^{+/+}$ and $\alpha 7^{-/-}$ EAE mice
 Wild type ($\alpha 7^{+/+}$) or nAChR $\alpha 7$ subunit knock-out ($\alpha 7^{-/-}$) EAE mice were immunized with MOG/CFA and treated with nicotine (Nic) or PBS. Mice were sacrificed on day 11 post-immunization plus nicotine/PBS treatment, and splenic (A, B) or CNS (C, D) mononuclear cells were isolated as described in the *Materials and Methods* section. A and C, representative dot plots of flow cytometry results for CD4⁺, CD8⁺, CD3⁺ and CD3⁻CD19⁺ cells obtained from the spleen (panel A) or CNS (panel C). B and D, Absolute numbers (ordinate; mean $\pm$ SEM) are shown of CD4⁺, CD8⁺, CD3⁺ and CD3⁻CD19⁺ cells from spleen (panel B, 10⁵) or CNS (panel D, 10²) samples from PBS-treated $\alpha 7^{+/+}$ (solid bars), nicotine-treated $\alpha 7^{+/+}$ (open bars), PBS-treated $\alpha 7^{-/-}$ (gray bars) or nicotine-treated $\alpha 7^{-/-}$ (cross-hatched bars) EAE mice. Asterisks denote significant differences between indicated groups (n = 6 per group; * = p<0.05, ** = p<0.01). CNS cells were isolated from 10–12 mice and pooled. Dot plots are representative of 3 similar experiments. Nicotine modulates the distribution of lymphocyte subpopulations in the spleen and their recruitment to the CNS of EAE mice, partially via the $\alpha 7$ -nAChR subtype.

Fig. 3. Nicotine inhibits splenic cell proliferation partially via the $\alpha 7$ -nAChR subtype

Wild type ($\alpha 7^{+/+}$) or nAChR $\alpha 7$ subunit knock-out ($\alpha 7^{-/-}$) EAE mice were immunized with MOG/CFA and treated with nicotine (Nic) or PBS. Mice were sacrificed on day 11 post-immunization plus initiation of nicotine/PBS treatment, and splenic mononuclear cells were isolated as described in the *Materials and Methods* section. A, Cells were then cultured in the absence of Ag (Medium) or in the presence of MOG₃₅₋₅₅ or Con A, and splenic cell proliferation was measured via [³H]thymidine incorporation (10^3 cpm $\pm$ SEM; ordinate) for PBS-treated $\alpha 7^{+/+}$ (solid bars), nicotine-treated $\alpha 7^{+/+}$ (open bars), PBS-treated $\alpha 7^{-/-}$ (gray bars) or nicotine-treated $\alpha 7^{-/-}$ (cross-hatched bars) EAE mice (* = $p < 0.05$, ** = $p < 0.01$, † = $p < 0.06$). B, In a separate set of experiments, splenic cells obtained from nicotine- or PBS-treated $\alpha 7^{+/+}$ or $\alpha 7^{-/-}$ EAE mice were cultured in the presence of MOG₃₅₋₅₅ and various concentrations of nicotine (0.1, 1, 10 or 100 μ M), and [³H]-thymidine incorporation was measured (10^3 cpm $\pm$ SEM; ordinate) for samples from PBS-treated $\alpha 7^{+/+}$ (●), nicotine-treated $\alpha 7^{+/+}$ (▲), PBS-treated $\alpha 7^{-/-}$ (▼) or nicotine-treated $\alpha 7^{-/-}$ (○) EAE mice (n = 6–8 per group; * represents a concentration-dependent effect, $p < 0.05$, at 10 μ M nicotine compared to lower concentrations; ** represents significant differences, $p < 0.05$, at all concentrations compared to other treatment groups). These data demonstrate that nicotine inhibits mononuclear cell proliferation in EAE mice, even in the absence of $\alpha 7$ -nAChR.

Fig. 4. Effects of nicotine on the differentiation and recruitment of regulatory T cells in $\alpha 7^{+/+}$ and $\alpha 7^{-/-}$ EAE mice

Wild type ($\alpha 7^{+/+}$) or $\alpha 7$ nAChR knock-out ($\alpha 7^{-/-}$) EAE mice were immunized with MOG/CFA and treated with nicotine (Nic) or PBS. Mice were sacrificed on day 11 post-immunization plus nicotine/PBS exposure, and mononuclear cells were isolated from the lymph nodes and CNS as described in the *Materials and Methods* section. A, Representative plots of CD25 and FoxP3 expression, gated on CD4⁺ cells from the CNS (upper row) or spleen (bottom row). B and C, The percentage of cells that are CD4⁺CD25⁺ (Tregs) and that also exhibit FoxP3 expression was quantified (% of spleen or CNS cells; mean $\pm$ SEM; ordinate) in the spleen (panel B) or CNS (panel C) from PBS-treated $\alpha 7^{+/+}$ (solid bars), nicotine-treated $\alpha 7^{+/+}$ (open bars), PBS-treated $\alpha 7^{-/-}$ (gray bars) or nicotine-treated $\alpha 7^{-/-}$ (cross-hatched bars) EAE mice. D, Percentage of CD4⁺ cells (ordinate) that also express FoxP3. Note that nicotine significantly increases the proportions of FoxP3⁺ Tregs in the periphery and more notably in the CNS during EAE, an effect that is partially retained in $\alpha 7^{-/-}$ mice (n = 6–8 per group; * = p<0.05, ** = p<0.01, † = p<0.06).

Fig. 5. Nicotinic modulation of myeloid cell recruitment and expression of co-stimulatory proteins in $\alpha 7^{+/+}$ and $\alpha 7^{-/-}$ EAE mice

Wild type ($\alpha 7^{+/+}$) or nAChR $\alpha 7$ subunit knock-out ($\alpha 7^{-/-}$) EAE mice were immunized with MOG/CFA and treated with nicotine (Nic) or PBS. PBS-treated $\alpha 7^{+/+}$ (solid bars), nicotine-treated $\alpha 7^{+/+}$ (white bars), PBS-treated $\alpha 7^{-/-}$ (gray bars) or nicotine-treated $\alpha 7^{-/-}$ (cross-hatched bars) mice were sacrificed on day 11 post-immunization plus nicotine/PBS treatment, and splenic or CNS mononuclear cells were isolated as described in the *Materials and Methods* section. A, Absolute numbers ($\times 100 \pm \text{SEM}$; ordinate) of microglia/macrophages (CD11b⁺) in CNS samples are shown. B and C, Percent ($\pm \text{SEM}$; ordinate) of CD11b⁺ cells that express co-stimulatory proteins MHC-II, B7.1 (CD80) and B7.2 (CD86) in the spleen (panel B) or CNS (panel C). D, Absolute numbers ($\times 100 \pm \text{SEM}$; ordinate) of dendritic cells (CD11c⁺) in CNS samples were quantified. E and F, Percent ($\pm \text{SEM}$; ordinate) of spleen or CNS CD11c⁺ cells that express MHC-II, CD80 or CD86. These results suggest that nicotine modulates the infiltration of CD11b⁺ and CD11c⁺ cells into the CNS and the expression of MHC-II and CD80 on CD11b⁺, but not CD11c⁺, cells via multiple nAChR subtypes including $\alpha 7$ -nAChRs ($n = 6-8$ per group; * = $p < 0.05$, ** = $p < 0.01$, † = $p < 0.06$).

Fig. 6. Expression of nAChR subunit mRNAs

Microglia (M), CD3⁺CD4⁺ (CD4⁺), CD3⁺CD8⁺ (CD8⁺), CD45⁺CD11b⁺ (CD11b⁺), CD45⁺CD11c⁺ (CD11c⁺) or CD4⁺CD25⁺FoxP3⁺ (T_{reg}) cells from naïve or MOG^{35–55}-immunized α7^{+/+} and α7^{-/-} mice were isolated by FACS (~1×10⁶ cells per sample, n = 4–8 per group) as described in the methods section. mRNA was purified from these cells, pooled, and cDNA was synthesized by reverse transcription. PCR was then performed using primers specific for each mouse nAChR subunit. A positive control (B), using whole mouse brain cDNA, and a negative control without cDNA (not shown), were included in each reaction. For each nAChR subunit, bands from the positive control and at least one sample from microglia were sequenced and confirmed as the correct PCR product. Microglial cells therefore express mRNA for all nAChR subunits, including the α7 subunit, whereas CD4⁺ and CD8⁺ T cells, monocytes/macrophages and dendritic cells have differential expression for various nAChR subunits. The only nAChR mRNAs consistently found in all samples were for the α9 and β2 subunits. Of note, nAChR α6 (in CD4⁺ T cells), α7 (in CD4⁺ and CD8⁺ T cells) and β4 (in CD4⁺ T cells) mRNAs were upregulated in MOG_{35–55}-immunized mice, whereas nAChR α5 (in CD11c⁺ cells) and α7 (in CD11b⁺ and CD11c⁺ cells) subunit messages were downregulated in EAE mice. The only compensatory change observed in α7^{-/-} mice was a decrease in nAChR α4 mRNA levels in CD8⁺ T cells. Although PCR was only performed in one Treg cell sample consisting of CD4⁺CD25⁺FoxP3⁺ cells pooled from 8 naïve α7^{+/+} animals, our results suggest that these cells do not express mRNA for any nAChR subunit assessed in this study.

Table 1

Primers used to detect nAChR subunits.

nAChR Subunit	NCBI Template	Forward Primer Sequence	Reverse Primer Sequence	Product Size (bp)	Tm (°C)
$\alpha 3$	NM_145129.2	gccaacctcacagaagctc	atgtgggttttagcagcaac	680	58
$\alpha 4$	NM_015730.5	cagtagccaataatctcagat	gtagaacacagtgaggcagtcgg	577	55
$\alpha 5$	NM_176844.3	gggttcgtcctcttggaacacctga	ggctctgtaggattatctcg	431	58
$\alpha 6$	NM_021369.2	tgtccacagcagataaacatctg	tgaattgaacactctcgtatg	987	55
$\alpha 7$	NM_007390.3	cgtagggcctctcagtggtctcg	ggccatgagggcacaaagcgggt	514	58
$\alpha 9$	NM_001081104.1	ctatttcccttcgacag	tttttcagtgcttcacagc	798	55
$\beta 2$	NM_009602.3	ctccaaactctatggcgtgct	cgtcggcctggcagtgcgat	623	58
$\beta 3$	NM_173212.3	acggagagtaagggaaccgt	accagcagccctcagttcta	364	58
$\beta 4$	NM_148944.4	tcctgttcgctctgcttca	acacagtggtgacgatggaa	913	58

Primer pairs were designed with the use of PubMed's "Primer-blast" tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). For each pair, forward and reverse primers were specific to different exons, so that potential DNA contamination could be ruled out. The optimal annealing temperature was determined empirically with the use of a temperature gradient thermocycler, while maintaining high stringency conditions. PCR conditions were the following: 95 °C for 5 minutes, followed by 35–40 cycles of 95 °C for 1 minute, 55–58 °C for 1 minute and 72 °C for 1.5 minutes, and a final extension step of 72 °C for 10 minutes. PCR products for each primer pair were sequenced in order to ensure the accuracy of our results.

Table 2

Cytokine secretion measured by ELISA.

Cytokine	$\alpha 7^{+/+}$ PBS	$\alpha 7^{+/+}$ Nic	$\alpha 7^{-/-}$ PBS	$\alpha 7^{-/-}$ Nic
IFN- γ (ng/ml)	210 $\pm$ 40	64 $\pm$ 3.0*	156 $\pm$ 39	96 $\pm$ 12**
IL-10 (pg/ml)	80 $\pm$ 19	171 $\pm$ 40*	100 $\pm$ 27	126 $\pm$ 21**
TGF- β (pg/ml)	120 $\pm$ 36	252 $\pm$ 46*	131 $\pm$ 26	144 $\pm$ 42
IL-2 (pg/ml)	452 $\pm$ 51	301 $\pm$ 42*	458 $\pm$ 28	365 $\pm$ 36
IL-4 (pg/ml)	UT ^b	UT ^b	UT ^b	UT ^b
IL-17 (pg/ml)	191 $\pm$ 67	121 $\pm$ 62	160 $\pm$ 39	142 $\pm$ 27

Mice were immunized to induce acute EAE and treated with PBS or with nicotine at a dose of ~13 mg/kg daily for a total of 7 days. Mice were sacrificed 14 days after immunization, and splenic mononuclear cells were isolated and cultured in the presence of MOG (see *Materials and Methods*). Supernatants were harvested after 36 h, and IFN- γ , IL-17, IL-2, IL-10 or TGF- β 1 secretion was determined by ELISA. Data are presented as means $\pm$ SD of values for 9 mice per group;

* $p < 0.05$ vs. $\alpha 7^{+/+}$ PBS,

** $p < 0.05$ vs. $\alpha 7^{-/-}$ PBS.

^bUT: Undetectable