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Activation of the Cholinergic Anti-Inflammatory System by Nicotine Attenuates Neuroinflammation via Suppression of Th1 and Th17 Responses

Eran Nizri,* Michal Irony-Tur-Sinai,* Omer Lory,* Avi Orr-Urtreger,† Ehud Lavi,‡ and Talma Brenner1*

The α7 nicotinic acetylcholine receptor (nAChR) was recently described as an anti-inflammatory target in both macrophages and T cells. Its expression by immune cells may explain the epidemiological data claiming a negative link between cigarette smoking and several inflammatory diseases. In this study, we determined the immunological effects of α7 nAChR activation by nicotine. Our results indicate that the α7 nAChR is expressed on the surface of CD4+ T cells and that this expression is up-regulated upon immune activation. Nicotine reduced T cell proliferation in response to an encephalitogenic Ag, as well as the production of Th1 (TNF-α and IFN-γ) and Th17 cytokines (IL-17, IL-17F, IL-21, and IL-22). IL-4 production was increased in the same setting. Attenuation of the Th1 and Th17 lineages was accompanied by reduced T-bet (50%) and increased GATA-3 (350%) expression. Overall, nicotine induced a shift to the Th2 lineage. However, α7−/−-derived T cells were unaffected by nicotine. Furthermore, nicotine reduced NF-κB-mediated transcription as measured by IL-2 and IκBα transcription. In vivo, administration of nicotine (2 mg/kg s.c.) suppressed the severity of CD4+ T cell-mediated disease experimental autoimmune encephalomyelitis. α7−/− mice were refractory to nicotine treatment, although disease severity in those animals was reduced, due to impairment in Ag presentation. Accordingly, CD4+ and CD11b+ cells infiltration into the CNS, demyelination, and axonal loss were reduced. Our data implicate a role for the α7 nAChR in immune modulation and suggest that α7 nAChR agonists may be effective in the treatment of inflammatory disorders. The Journal of Immunology, 2009, 183: 6681–6688.

The presence of a complete cholinergic system on immune cells, consisting of muscarinic acetylcholine receptors and nicotinic acetylcholine receptors (nAChRs), and the ability to synthesize and degrade acetylcholine (ACh) are well documented (1). The α7 nAChR is a homopentameric nAChR present on macrophages (2), T cells (3, 4), and B cells (5). It was shown that activation of this receptor on macrophages reduces secretion of the proinflammatory cytokines TNF-α, IL-1β, and IL-6, whereas production of anti-inflammatory cytokines such as IL-10 is not affected (6). Signaling downstream to α7 nAChR in this system involves the NF-κB pathway (7), and vagal innervation was implicated as the source of ACh for the immune cells (8). A previous study done in our laboratory showed that pharmacological activation of α7 on T cells has similar anti-inflammatory effects, highlighting the relevance of the cholinergic anti-inflammatory pathway also in adaptive immunity (3, 9). However, the specific mechanisms by which α7 nAChR activation regulates the immune response are not clearly defined.

Epidemiological data indicate that smokers have a relatively lower incidence of inflammatory diseases such as ulcerative colitis (UC) and sarcoidosis (10). Animal studies defined nicotine as the active substance in cigarette smoke responsible for this immunosuppression and implicated various mechanisms in the process, including decreased Ab production and signaling through the TCR (11, 12). It is tempting to speculate that at least part of the effects of nicotine on the immune system stem from α7 nAChR-mediated activation of these cells.

Experimental autoimmune encephalomyelitis (EAE) is a CNS inflammatory disease in which myelin components are the focus of the autoimmune attack. Myelin-sensitized T cells penetrate into the CNS and cause neurological deficit that can be quantified according to clinical and pathological parameters (13). In this study, we resorted to myelin oligodendrocyte glycoprotein (MOG)35-55-induced EAE, because it is characterized by an acute inflammatory phase, followed by a chronic phase of neurological deficit. This pattern is more reminiscent of the human disease multiple sclerosis, the disease recapitulated by this model (14).

Until recently, EAE was mainly considered a Th1-mediated disease; however, experimental evidence, including increased EAE severity in IFN-γ-deficient mice and the inability of anti-IFN treatment to ameliorate the disease, led to reassessment of this concept (15). Contemporary research has emphasized the importance of Th17 lineage, a novel Th lineage, in EAE and autoimmune in general (16–19). Th17 cells secrete a specific set of cytokines (IL-17, IL-17F, IL-21, and IL-22) (20), have specific lineage transcription factors (TFs), namely ROR-γT (21) and ROR-α (22), and develop in a unique cytokine milieu (23). However, it seems that even in the Th17 era, the role of Th1 cells in autoimmune pathogenesis cannot be ignored (24). It was recently shown that both
Th1 and Th17 T cells can mediate EAE when adoptively transferred (25). Although the clinical severity of the disease did not differ between the two Th lineages, the cellular constituents of the inflammatory infiltrate in the CNS did. Moreover, the fact that Th1-induced EAE did not respond to anti-IL-17 treatment, as opposed to Th17-induced EAE, supports the presence of different and independent pathways exerting autoimmunity. In experimental autoimmune uveitis, it was also shown that whereas IL-17 is necessary in the actively induced model with adjuvant immunization, it was dispensable in the adoptive transfer model (26). Th1-polarized cells alone could mediate the disease in that case, and adaptive transfer of Ag-pulsed dendritic cells induced uveitis only in animals capable to mount a Th1 response (26). Hence, when analyzing immunomodulatory treatment for EAE, the immunological effects on both lineages should be addressed.

Our data show that the α7 nAChR is expressed by CD4+ T cells and that this expression is up-regulated following T cell activation. Nicotine treatment decreased the reactivity of the Th1 and Th17 lineages and increased Th2, including both reduced specific cytokine production and expression of lineage-specific TFs. These effects were not reproducible in α7−/−-deficient animals. Nicotinic activation caused reduced NF-κB transcription. Ultimately, nicotine administration significantly ameliorated the clinical course of EAE and reduced T cell and macrophages infiltration into the CNS. Overall, the results implicate a central role for the T cell α7 nAChR in immunological responses.

Materials and Methods

Flow cytometry analysis

For the staining of the α7 nAChR CD4+ cells were purified from naive WT mice spleens using MACS cell separation reagents (Miltenyi Biotec) and activated in the presence of 2.5 μg/ml Con A. We used FITC-conjugated α-bungarotoxin (α-btx; Molecular Probes), a cholinerigic nicotinic blocker, for α7 detection. Incubation with antisense (AS) to the α5 and the α7 subunits and the specific sequence of the AS were described previously (9). For leukocyte surface markers, pooled spleen cells were obtained from wild-type (WT) and knockout (KO) mice (as described in the mouse lymphocyte proliferation assay). Cell suspensions were prepared as described previously (27). For immune phenotyping, the following Abs were used: anti-CD4-FITC (clone GK1.5; BD Pharmingen), anti-CD8-FITC (clone 53-6.7; BD Pharmingen), anti-CD11b-FITC (clone M1/70; BD Pharmingen), anti-CD19-FITC (clone 1D3; BD Pharmingen), and anti-CD11c-PE (clone N418; Serotec). The following isotype controls were used: FITC-conjugated rat IgG2b (clone A 95-1; BD Pharmingen), FITC-conjugated rat IgG2a (clone R35-95; BD Pharmingen), and PE-conjugated hamster IgG (clone HTK888; Biologend). Stained cells were counted in a FACS (LSR; BD Biosciences).

EAE induction and treatment

EAE was induced in 8-wk-old female C57BL/6 mice by injecting s.c. into the left para-lumbar region 125 μg of MOG35–55 peptide (synthesized by Sigma-Aldrich) imubulated with CFA. Immediately thereafter, and again at 48 h, 0.5 ml of saline, corresponding to a daily dose of 2 mg/kg/day for 28 days. Control animals were sham operated, because previous work done in our laboratory indicated that saline-loaded pumps had no effect on disease course (9). α7−/− mice on C57BL/6 background were described previously (28). Induction of EAE and treatment with nicotine in these animals was similar to that in the WT animals.

FIGURE 1. Expression of α7 nAChR by CD4+ T cells. CD4+ T cells were isolated from naive C57BL mice and activated in the presence of 2.5 μg/ml Con A for 24 h and then stained with 2 μM FITC-labeled α-btx. A, Naive CD4+ cells express the α7 protein, and (B) expression is increased upon activation (filled gray, unstained cells; black line, α-btx-labeled cells). C, AS to the α5 nAChR mRNA (10 μM) did not significantly reduce α-btx staining. D, AS to α7 nAChR mRNA (10 μM) markedly reduced α-btx staining (filled black, unstained cells; black line, Con A activated; gray line, AS α5- or AS α7-treated cells). Staining of control cells (Con A activation without pre-incubation with AS) was 75.2%. The results of a representative experiment out of two is shown (n = 4).

Mouse lymphocyte proliferation assay

Pooled lymph node cells (LNCs) were prepared from inguinal, axillary, and mesenteric lymph nodes from spleens of mice that had been inoculated 9 days earlier with MOG35–55 peptide in CFA, with or without treatment. The ex vivo response of the LNCs was assayed as described previously (27). Where indicated, CD4+ cells were separated from splenocytes, using MACS cell separation reagents. The results are expressed as stimulation index (SI), according to the following equation: SI = mean cpm of the stimulated cells/mean cpm of the unstimulated controls.

Analysis of cytokine production

The presence of IFN-γ, TNF-α, IL-17, IL-4, and IL-10 of was determined in the culture media of LNCs incubated in the presence of MOG35–55, using an ELISA kit (Biologend). The lymphocytes were collected from the mice 9 days after inoculation with MOG35–55 with or without nicotine treatment (2 mg/kg s.c.).

Preparation of RNA and cDNA

Total RNA was prepared using an SV total RNA kit (Promega). To determine the mRNA level of the various TFs, RNA was prepared from spleen cells of treated and untreated mice on day 9 after induction of EAE as described above. cDNA was prepared from 500 μg of total RNA, using murine leukemia virus reverse transcriptase (Applied Biosystems) and random hexamers according to the manufacturer’s instructions for first-strand cDNA synthesis.

Quantitative real-time PCR

The semiquantitative real-time PCR reaction was performed as described previously (3). The results for gene expression were normalized according to the glyoxalamine phosphoribosyltransferase (HPRT) gene. The primers used were: HPRT forward, CAGCCCAAATGGTTAAGGT, and HPRT reverse, CGAGAGGTCCTTTTCACCGC; T-bet forward, CAGTTC
ATTGCAGTGACTGCCTAC, and T-bet reverse, CAAAGTTCTCCCGGAATCCTTT; GATA-3 forward, GCAGAAAGCAAAATGTTTGCTTC, and GATA-3 reverse, GAGTCTGAATGGCTTATTCACAAATG; IL-17F forward, CCCAGGAAGACATACTTAGAAGAAA, and IL-17F reverse, CAACAGTAGCAAAGACTTGACCA; IL-21 forward, GACATTCATCATTGACCTCGTG, and IL-21 reverse, TCACAGGAAGGGCATTTAGC; and IL-22 forward, GACAGGTTCCAGCCCTACAT, and IL-22 reverse: GTCGTCACCGCTGATGTG.

Ag presentation assay

APCs were derived from spleens of WT or \(\alpha7^{-/-}\) naive mice. Before coculture with responder T cells, the splenocytes were irradiated with 3000 rad, so that proliferation in coculture assays is attributable solely to the responder T cells. Responder T cells were obtained from MOG35–55-stimulated LNCs derived from MOG-immunized WT mice. The cells were maintained in MOG-containing medium for 4 days, after which the medium was replenished and supplemented with IL-2 (20 \(\mu\)g/ml) for an additional 5 days. For the Ag presentation assay, 50,000 (per well) responder T cells were cocultured with 300,000 (irradiated) splenocytes derived from WT or \(\alpha7^{-/-}\) naive mice, in the presence of MOG (50 \(\mu\)g/ml) for 72 h. Cells were cultured in RPMI 1640 containing 10% FCS supplemented with 5 \(\times\) 10\(^{-5}\) M 2-ME, 1 mM sodium pyruvate, nonessential amino acids, \(\alpha\)-glutamine and 100 U of penicillin/100 \(\mu\)g streptomycin/ml. Responder T cells proliferation was assessed by \([3H]\) thymidine incorporation.

Isolation of inflammatory cells from CNS tissue

At the peak of the disease (day 18 after immunization), mice were anesthetized and perfused with cold PBS by the intracardiac route. Spinal cords and brains were removed and pooled for mononuclear cell isolation, according to a standard protocol. Briefly, tissues were minced and digested with 2 mg/ml collagenase (Worthington Biochemical) for 1 h at 37ºC. Pooled brain and spinal cord cells were layered over a discontinuous Per-}

FIGURE 2. Reduced T cell reactivity in EAE mice treated with nicotine. Miniosmotic pumps loaded with 2 mg/kg/day nicotine were implanted in the mice, and pooled lymphocytes were obtained 9 days post-EAE induction. A, Nicotine reduced T cell proliferation induced by encephalitogenic Ag MOG\(_{35-55}\). B, Nicotine reduced production of the Th1 lineage cytokines TNF-\(\alpha\) and IFN-\(\gamma\). C, It also reduced production of IL-17 and (D) other IL-17 lineage-related cytokines. E, Treatment with nicotine reduced IL-10 production but increased that of IL-4. F, Nicotine effects were determined on mRNA levels of Th1 and Th2 lineage-specific transcription factor: reduction in T-bet (Th1) and increase in GATA-3 (Th2) expression. The results summarize four different experiments (\(n = 8\) for each group). * \(p < 0.05\); ** \(p < 0.01\); *** \(p < 0.001\).
mitogenic stimulation (3, 9). To ascertain α7 nAChR protein expression (because chaperone proteins, such as RIC-3, are also necessary (31)), CD4+ T cells were labeled with FITC-conjugated α-btx, a nicotinic cholinergic blocker. Fig. 1A shows that 32% of unactivated CD4+ T cells express the α7 protein. Expression was up-regulated 24 h after activation with Con A (75%) (Fig. 1B). Incubation of CD4+ T cells with AS to α7 or α5 mRNA before activation ensured the specificity of staining. Fig. 1C shows that preincubation with AS α5 did not significantly affect the extent of staining (65.7 vs 75.2% for AS α5 and control, respectively). In contrast, preincubation with AS α7 markedly reduced α-btx-positive cells (26.4 vs 75.2% for AS α7 and control, respectively) (Fig. 1D). HEK293 cells, which express low levels of RIC-3 and α7mRNA, were not stained by the marker nor were α7(nAChR)-derived CD4+ T cells (data not shown).

Nicotine treatment is associated with reduced T cell reactivity

Lymphocytes obtained 9 days after immunization with MOG35-55 from mice treated with nicotine (2 mg/kg via miniosmotic pumps, see below) showed reduced proliferation in response to the Ag (Fig. 2A). Nicotine also inhibited production of the Th1 cytokines TNF-α and IFN-γ by 50% (Fig. 2B). The production of IL-17 was reduced as well (Fig. 2C). In addition, the mRNA levels of IL-17-related cytokines such as IL-17F, IL-21, and IL-22 were reduced by ~50% (Fig. 2D). Nicotine also inhibited the levels of IL-10, an anti-inflammatory cytokine in the context of EAE and increased by 2-fold the level of IL-4, the prototypic Th2 cytokine (Fig. 2E). We tested the effects of nicotine on the mRNA levels of specific TFs responsible for Th lineage differentiation. As can be seen in Fig. 2F, nicotine reduced by 50% T-bet mRNA levels, a pivotal Th1 TF (32), but increased by 350% the expression of GATA-3, a Th2 TF (33). The levels of ROR-γT and ROR-α were not changed despite the observed change in IL-17 and other IL-17-related cytokines (data not shown). Cumulatively, the results support the role of nicotine in skewing Th lineage development from Th1 and Th17 to Th2.

Involvement of α7 nAChR in the anti-inflammatory effect of nicotine

To prove the involvement of the α7 nAChR in the nicotine-induced reduction in T cell reactivity, the reactivity of splenocytes obtained from α7−/− mice (KO) toward MOG35-55 was assayed. Fig. 3A shows that the specific proliferation of the encephalitogenic cells was reduced in KO mice in comparison with that of the WT. The same was found for proinflammatory cytokine production (Fig. 3B): reduced overall secretion by T cells derived from α7−/− mice compared with that of the WT-derived cells. However, the level of IL-4 was increased in KO-derived T cells. These results were unforeseeable in view of the anti-inflammatory role of the α7 nAChR. We anticipated that its absence would be accompanied by increased proliferation and cytokine production. Nevertheless, the α7−/− mice were unresponsive to the suppressive effects of nicotine both on proliferation and cytokine production. Accordingly, whereas nicotine increased IL-4 production in WT mice (see Fig. 2E), it did not change this production in KO mice.
Nicotine reduces NF-κB-mediated transcription. Splenocytes were obtained from naive and activated in vitro with Con A-activated mice. Upon activation, NF-κB transcription was increased, as can be inferred from the increase in IκB and IL-2 transcription. Nicotine downregulated this transcription. The results summarize three different experiments run in triplicate, n = 3. *p < 0.05; **p < 0.01.

Thus, it seems that the effects of nicotine are α7 nAChR dependent. However, the effects of α7 deficiency may be attributed to its absence in APCs or, alternatively, in effector T cells. To discriminate between these possibilities, we tested Ag presentation by α7−/−-derived APCs and proliferation of α7−/−-derived CD4+ T cells. Fig. 3C shows that CD4+ T cells from KO mice exhibited greater proliferation in response to mitogenic activation by CD3/CD28 (SI = 42.2, SI = 66.2 for WT and KO, respectively; p < 0.05) or by Con A (SI = 109, SI = 140.1 for WT and KO, respectively; p < 0.001). CD4+ T cells from WT, but not KO, showed reduced proliferation in the presence of nicotine. However, Ag presentation by α7−/−-derived APCs was impaired. Fig. 3D shows that the capability of α7−/−-derived APCs to present MOG35−55 and to induce proliferation of the corresponding specific T cell clone was 25% less than that of WT-derived APCs. Thus, whereas α7 deficiency in CD4+ T cells increased their reactivity, its absence in APCs impaired their function.

Nicotine reduces NF-κB dependent transcription

NF-κB is a pivotal transcription factor in inflammatory activation. Among its many responsive genes are IL-2 and IFN-γ. We tested the expression of these genes to determine whether nicotine can affect NF-κB mediated transcription. Mitogenic activation in naive splenocytes is associated with activation of NF-κB and hence increased IL-2 and IκB transcription (Fig. 4). Nicotine markedly reduced transcription of IL-2 and IκB in this setting.

Treatment with nicotine reduces clinical severity of EAE

To evaluate the effects of α7 nAChR activation in vivo, EAE-induced mice were treated with nicotine delivered via miniosmotic pumps. Sustained release was selected due to nicotine’s short half-life (35), and the dosage chosen was 2 mg/kg/day. Under these conditions, no signs of toxicity were detected in the treated animals. Treatment with nicotine reduced clinical severity by 75% (Fig. 5A and Table I). It also reduced the number of inflammatory infiltrates on histopathological sections from 11.2 ± 1.7 to 2.3 ± 1.3 (p = 0.01, n = 8 for each group). A similar trend was observed when infiltrating CNS cells were analyzed by flow cytometry (Fig. 6) at the peak of the disease. Nicotine administration blocked almost completely the infiltration of CD4+ cells (46.6 and 5.2% for control and nicotine, respectively). It also reduced the number of CD11b+ cells in the CNS (23.2 and 15.5%, for control and nicotine, respectively). Histopathological analysis of spinal cords from both groups demonstrated tissue preservation upon nicotine treatment (Fig. 7). This was manifested as decreased demyelination (Fig. 7, C and D) and microglia infiltration (Fig. 7, B and E) and culminated in reduced axonal damage (Fig. 7, F).

Nicotine is known to increase corticosteroids release by activation of the hypothalamic-pituitary-adrenal axis (36). To exclude control and nicotine, respectively). It also reduced the number of CD11b+ cells in the CNS (23.2 and 15.5%, for control and nicotine, respectively). Histopathological analysis of spinal cords from both groups demonstrated tissue preservation upon nicotine treatment (Fig. 7). This was manifested as decreased demyelination (Fig. 7, C and D) and microglia infiltration (Fig. 7, B and E) and culminated in reduced axonal damage (Fig. 7, F).

Nicotine treatment ameliorates clinical parameters of EAE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean disease severity</th>
<th>Cumulative score</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>3.3 ± 0.4</td>
<td>70 ± 8.2</td>
</tr>
<tr>
<td>Nicotine</td>
<td>0.8 ± 0.1***</td>
<td>17 ± 7.1***</td>
</tr>
<tr>
<td>Nicotine (ADEX)</td>
<td>0.6 ± 0.1***</td>
<td>9.8 ± 3.9***</td>
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*The data are expressed as the mean ± SE. Miniosmotic pumps were implanted s.c. in mice treated with nicotine and the dosage was set at 2 mg nicotine/kg/day. Control mice were sham operated. **p < 0.001 vs. control.

ADEX, adrenalectomized mice.

Cumulative score: cumulative clinical score of days animals were sick.
the possibility that the effects of nicotine on EAE depend on corticosteroids, EAE in adrenalectomized mice was treated with nicotine. As can be seen in Fig. 5A, the effects of nicotine were not dependent on intact adrenal function, because EAE was inhibited to the same extent in the adrenalectomized mice. These results are in accordance with previous published results regarding chronic nicotine treatment (11).

To determine whether the \( \alpha_7 \) subunit is required for nicotine to exert its effects on EAE, the disease was induced in \( \alpha_7^{-/-} \) mice. First, the course and severity of EAE on this genetic background was studied. Fig. 5B shows that \( \alpha_7^{-/-} \) mice developed EAE of milder severity in comparison with that in the wild-type mice (by \( \sim 35\% \), \( p < 0.001 \); see Table II). This result is in accordance with the suppressed proliferation and cytokine production by T cells derived from these animals in response to MOG35–55. However, when these animals were treated with nicotine, the treatment was ineffective. This demonstrates that the effect of nicotine on EAE course depends on \( \alpha_7 \) nAChR. It should be noted that analysis of the distribution of various peripheral immune cells in EAE-immunized WT and in \( \alpha_7^{-/-} \) mice revealed no difference in the number of Th cells, CTLs, macrophages, and dendritic cells (Table III).

### Discussion

The purpose of this study was to investigate whether the \( \alpha_7 \) nAChR is involved in the anti-inflammatory effects of nicotine and to delineate the specific immunological mechanisms involved in this effect.

<table>
<thead>
<tr>
<th>( \alpha_7^{-/-} )</th>
<th>( \alpha_7^{-/-} + \text{Nicotine} )</th>
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<tbody>
<tr>
<td>Mean disease severity</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Cumulative score*</td>
<td>32 ± 5.9</td>
</tr>
</tbody>
</table>

* Cumulative score: cumulative clinical score of days animals were sick.

FIGURE 6. Nicotine treatment reduces CNS inflammatory cell infiltration. Infiltrating cells were separated from brains and spinal cords of control and nicotine-treated animals at the peak of the disease (day 18) and analyzed by flow cytometry. Note the marked reduction in CD4\(^+\) and CD11b\(^+\) cells in the CNS following nicotine treatment. The results are from one representative experiment out of two; \( n = 10 \) for each group.

FIGURE 7. Treatment with nicotine improves histopathological parameters of EAE. Spinal cords of mice from the control and nicotine groups were removed at the chronic phase of the disease (day 30) and analyzed for demyelinization (A and D), microglia activation (B and E) and axonal damage (C and F). A–C, Untreated group; D–F, nicotine-treated group; \( n = 7 \) for each group.
The main findings of this study are: 1) α7 nAChR activation by nicotine has immunomodulatory properties, suppressing Th1 and Th17 reactions but not Th2. 2) In vivo treatment with nicotine of mice with intact α7 nAChR markedly suppressed EAE clinical symptoms and inflammatory CNS infiltration.

CD4+ T cells express an α-btX binding site on their surface, which may be up-regulated following T cell activation. α-btX binds both to the neuronal nAChR (e.g., α7 and α9) and the muscle-type nAChR. However, we and others (3, 4) have shown that T cells do not express mRNA of muscle-type nAChR subunits nor of the α9 subunit (another neuronal nAChR), so that the α-btX staining of these cells probably reflects α7 nAChR protein expression. The finding that such staining was increased in activated cells supports the pharmacological use of α7 agonists in inflammatory conditions, because activated cells would be more responsive to these agents.

Nicotine treatment reduced encephalitogenic T cell proliferation and the production of Th1 and Th17 cytokines in response to MOG35–55 in WT animals. At the same time, levels of IL-10 were reduced, whereas levels of IL-4, the main Th2 cytokine, were increased. Reduced production of IL-17 related cytokines. It is possible that nicotine treatment affects Th17 T cells but not their differentiation/maturation. Alternatively, the reduced production of IL-17 related cytokines may be explained by the inhibition of T-bet transcription as this TF is necessary for their optimal production (16, 26). Similar to the signaling pathway described in macrophages (7), our results show that α7 activation on T cells is also associated with reduced NF-κB transcription.

T cells derived from α7−/− mice did not respond to nicotine. T cell proliferation, Th1, Th17, and Th2 reactivity were unchanged. However, α7−/− mice present a complex phenotype with reduced T cell proliferation and cytokine production in response to MOG35–55. Accordingly, the severity of EAE was reduced in α7 KO mice. There was no change in specific immune cell populations that could account for this finding, because the distribution of these populations was similar between the genotypes. These results are somewhat unexpected since the lack of this anti-inflammatory target would be expected to induce increased T cell reactivity and EAE severity. However, these findings are in accordance with a recently published study, which reported that EAE was not aggravated by the α7−/− phenotype (39). Overall clinical severity was similar in WT and α7−/− mice, with lower incidence in α7−/− mice (39). Thus, this study reinforces the notion that α7 deficiency does not aggravate disease severity as theoretically expected. It should be noted that the EAE induction protocol consisted of MOG1–125 immunization. In this specific model, Abs play a significant role in pathogenesis (39), which is not the case in the MOG35–55-induced EAE used in our study (40). However, analysis of T cell infiltration in the facial axotomy model showed increased T cell infiltration into the facial nucleus of α7−/− mice (39). As in this model reactivation of T cells in the CNS does not occur, in contrast to EAE, it is tempting to speculate that the increased infiltration of T cells in this model in α7−/− mice may represent the inhibitory effect of α7 nAChR on T cells.

It appears that α7 deficiency affects immune cell reactivity in a more complex way: while isolated T cells respond by a higher proliferation rate to stimulation, Ag presentation was found to be impaired. Accordingly, cytokine production by KO-derived T cells in response to the MOG peptide was reduced. Thus, the impairment in Ag presentation may explain the reduced T cell reactivity and EAE severity. The absence of α7 in APCs resulted in decreased Ag presentation, implicating a stimulatory, proinflammatory role for α7 activation and nicotine influence on APCs. A similar effect of nicotine on APCs was previously described (41), with increased dendritic cell reactivity and Ag presentation following exposure to nicotine. However, there are reports on the inhibition of Ag presentation of dendritic cells and disruption of their ability to induce Th1 lineage differentiation (37, 42) by nicotine. Whether our data regarding the role of nicotine in Ag presentation represent the normal situation or if this is a phenotypic compensation that occurs in transgenic animals (43) is unknown.

Nicotine administration markedly ameliorated EAE clinical symptoms alongside with reduced CD11b+ and CD4+ infiltration into the CNS. The histopathological findings correlated with the improvement in disease severity with less tissue destruction and increased neuronal preservation in treated mice. The effect of nicotine was not dependent on corticosteroid secretion because adrenalectomized mice responded like intact ones.

Sustained release was chosen due to the short half-life of nicotine, but it also has a human correlate—nicotine transdermal patches, which have been tried in clinical trials in doses of up to 25 mg/kg (44). Thus, on the one hand, the dose we used (2 mg/kg) enables an increment in resistant or fulminant inflammation but, on the other hand, may reduce adverse effects reported in these trials and increase safety.

Treatment with nicotine was tested clinically in UC, a disease known to be negatively associated with nicotine (35). Initial trials showed the efficacy of nicotine delivered in transdermal patches in controlling active disease (44, 45), but its failure to maintain remission (35). Meta-analysis of clinical data led to the conclusion that nicotine is effective in the control of active disease in comparison to placebo but is not superior to conventional medical therapy. However, its use in nonsmokers is confounded by its side effects (46). Thus, clinical data support an anti-inflammatory role for nicotine. Our results support the possibility that these effects are dependent on α7 activation of CD4+ T cells. It should be noted that the inflammatory process in UC is considered a Th2 process (47–49). Our data show that nicotine inhibited Th1 and Th17 immune responses but increased Th2. We speculate that nicotine may suppress any active immune clone, because these cells will express higher levels of the α7, as shown here for activated T cells. Indeed, preliminary results obtained in our laboratory indicate that differentiation and IL-4 production by Th2 cells can be suppressed by nicotine. Hence, nicotine suppressive effects are not dependent on the specific immune response nature but on the availability of α7 receptors. It should also be noted that there is a higher incidence of several other inflammatory conditions such as rheumatoid arthritis, atherosclerosis, peptic ulcer disease, and notably Crohn’s disease (10) in smokers. Apparently, in these situations, nicotine does not decrease inflammation. The reason for this complex response to nicotine is unknown. Further study of specific α7 agonists may elucidate the relative role of α7 nAChR and other nAChR during inflammation.

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Table III. Analysis of immune cell markers in splenocytes derived from C57BL WT and α7−/− mice

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>WT (%)</th>
<th>α7−/− (%)</th>
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<tbody>
<tr>
<td>CD4+ (Th)</td>
<td>6.5</td>
<td>7.5</td>
</tr>
<tr>
<td>CD8+ (CTLs)</td>
<td>10.7</td>
<td>9</td>
</tr>
<tr>
<td>CD11b+ (macrophages)</td>
<td>31.2</td>
<td>32</td>
</tr>
<tr>
<td>CD11c− (dendritic cells)</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>CD19− (B cells)</td>
<td>41.6</td>
<td>41.5</td>
</tr>
</tbody>
</table>

Splenocytes of WT and α7−/− mice were analyzed 9 days after immunization with MOG35–55. The results summarize two different experiments; n = 4 for each group.
The results presented in our study highlight the importance of α7 nAChR activation in immune responses and its ability to affect immunomodulatory processes. This central role may be explained by the fact that calcium signals have a pivotal role in the differentiation of specific T cell lineages. Sustained calcium influx is associated with Th1 differentiation, whereas transient influx directly differentiates the Th2 lineage (50). The rapid inactivation of the α7 nAChR is consistent with this theory (42). Another explanation for the pivotal role of α7 steps from the recent findings that this receptor is colocalized with the TCR (51). Cumulatively, our data indicate that α7 activation may prove to be a novel pathway for immunomodulation.

Disclosures

The authors have no financial conflict of interest.

References