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# Distinctive Roles for $\alpha 7^*$ - and $\alpha 9^*$ -Nicotinic Acetylcholine Receptors in Inflammatory and Autoimmune Responses in the Murine Experimental Autoimmune Encephalomyelitis Model of Multiple Sclerosis

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Previous studies have demonstrated immunosuppressive and anti-inflammatory effects of nicotine, including in the experimental autoimmune encephalomyelitis (EAE) model in mice of some forms of multiple sclerosis (MS). Other studies using knock-out (KO) mice have implicated nicotinic acetylcholine (ACh) receptors containing  $\alpha 7$ ,  $\alpha 9$ , or  $\beta 2$  subunits ( $\alpha 7^*$ -,  $\alpha 9^*$ - or  $\beta 2^*$ -nAChR) in different, disease-exacerbating or disease-ameliorating processes. These outcomes are in harmony with gene expression analyses showing nAChR subunit mRNA in many classes of immune system cell types. Consistent with influences on disease status, predictable effects of nAChR subunit (and subtype) KO, or of nicotine exposure, are seen on immune cell numbers and distribution and on cytokine levels or other markers of immunity, inflammation, demyelination, and axonal degradation. Providing support for our hypotheses about distinctive roles for nAChR subtypes in EAE, here we have used direct and adoptive EAE induction and a nAChR subunit gene double knock-out (DKO) strategy. Immune cell expression of nAChR  $\alpha 9$  subunits as protein is demonstrated by immunostaining of isolated CD4<sup>+</sup>, CD8<sup>+</sup>, CD11b<sup>+</sup> and CD11c<sup>+</sup> cells from wild-type (WT) mice, but not in cells from nAChR  $\alpha 9$  subunit KO animals. Nicotine exposure is protective against directly-induced EAE in WT or  $\alpha 7/\alpha 9$  DKO animals relative to effects seen in WT/vehicle-treated mice, but, remarkably, EAE is exacerbated in vehicle-treated  $\alpha 7/\alpha 9$  DKO mice. Brain lesion volume and intracranial inflammatory activity similarly are higher in DKO/vehicle than in WT/vehicle-treated animals, although nicotine's protective effects are seen in each instance. By contrast, in adoptive transfer studies, disease severity is attenuated and disease onset

**Abbreviations:**  $\alpha 7$  or  $\alpha 9$  KO, nicotinic acetylcholine receptor  $\alpha 7$  or  $\alpha 9$  subunit knock-out; ACh, acetylcholine; DKO, double knock-out; EAE, experimental autoimmune encephalomyelitis; FACS, fluorescence-activated cell sorting; KO, knock-out; MOG, myelin oligodendrocyte glycoprotein; MRI, magnetic resonance imaging; MS, multiple sclerosis; nAChR, nicotinic acetylcholine receptor(s); PBS, phosphate-buffered saline; ROS, reactive oxygen species; WT, wild-type.

is delayed in recipients of splenocytes from WT animals treated with nicotine rather than with vehicle. Moreover, protection as seen in nicotine-treated WT animals is the same in recipients of splenocytes from nAChR  $\alpha 7/\alpha 9$  DKO mice irrespective of their exposure to nicotine or vehicle. When combined with previous observations, these findings are consistent with disease exacerbation (or even induction) being mediated at least in part via  $\alpha 9^*$ -nAChR in peripheral immune cells. They also suggest protective roles of central nervous system (CNS)  $\alpha 7^*$ -nAChR. The results suggest that both  $\alpha 7^*$ - and  $\alpha 9^*$ -nAChR are potential targets of therapeutic ligands to modulate inflammation and autoimmunity.

**Keywords:** auto-immunity, cholinergic anti-inflammatory pathway, experimental autoimmune encephalomyelitis, inflammation, multiple sclerosis, nicotinic acetylcholine receptors

## INTRODUCTION

It is widely recognized and undisputed that controlled inflammatory and immune responses play critical roles in maintenance of normal health by protecting against insults, in part by clearing damaged cells, compromised tissue areas and/or foreign substances (Weiner and Selkoe, 2002; Nikoopour et al., 2008; Viganò et al., 2012). Conversely, aberrant or excessive inflammatory or immune responses and anti-self, autoimmune activities contribute to or literally cause a variety of diseases, such as inflammatory bowel disease, arthritis and multiple sclerosis (MS; Wekerle, 1998; Davidson and Diamond, 2001; Weiner and Selkoe, 2002; Nikoopour et al., 2008; Franklin et al., 2012; Pierson et al., 2012; Viganò et al., 2012; Lassmann, 2013; Laveti et al., 2013). Our work reported here sought to illuminate new bases for possible improvements in treatment of MS and other diseases provoked by inflammation and hyper-immunity.

We and others have shown protection against inflammation and immune hyper-responsiveness in the murine experimental autoimmune encephalomyelitis (EAE) model of some forms of MS upon sustained exposure to nicotine (Nizri et al., 2009; Shi et al., 2009). These findings are consistent with other, prior work, indicating that nicotine exposure inhibits, for example, T cell differentiation (Sato et al., 1999; Matsunaga et al., 2001; Kuo et al., 2002; Middlebrook et al., 2002; Kawashima et al., 2007; Fujii et al., 2008), with long-recognized indications that nicotine has anti-inflammatory and immunosuppressive effects (Ulloa, 2005; Cloëz-Tayarani and Changeux, 2007; Filippini et al., 2012), and with the concept of a cholinergic anti-inflammatory system (Tracey, 2009), which is postulated to suppress inflammatory and immune responses by integrating signaling in the immune and nervous systems.

Our subsequent studies have confirmed those protective effects in the EAE model and have illuminated roles for different nicotinic acetylcholine receptor (nAChR) subtypes in specific features of the disease process or recovery and in protective effects of nicotine exposure (Piao et al., 2009; Shi et al., 2009; Hao et al., 2011, 2013; Simard et al., 2013). nAChR exist as pentamers of nAChR subunits that are encoded from a mammalian family of 16 different genes (Jensen et al., 2005; Lukas and Bencherif, 2006; Taly et al., 2009). nAChR thus also exist as a group of subtypes, each defined by their distinctive subunit composition. Subtypes have unique distributions across body organs, organ (brain) regions, cell types and even across sub-cellular domains

(nerve cell soma, dendrites or axon terminals; pre-, post- or peri-synaptic), and each nAChR subtype also has unique features and characteristic responsiveness to acetylcholine (ACh) and nicotine. nAChR nomenclature is based on subunit composition to the extent that it is known or inferred, and an “\*” is used to indicate a set of nAChR containing the specified subunits but that additional and unspecified subunits may be or are known to be partners in the receptor assembly (Lukas et al., 1999).

Prior studies pointed to important roles for  $\alpha 7^*$ -nAChR in the cholinergic anti-inflammatory pathway (Wang et al., 2003; Shytle et al., 2004). Studies by us and others at first seemed consistent with a dominant role for  $\alpha 7^*$ -nAChR in protective effects of nicotine in EAE (Nizri et al., 2009; Hao et al., 2011). In part, this is because the integrated disease response, assessed based on signs of limb and tail weakness in the EAE model, was attenuated by nicotine treatment in wild-type (WT) animals, but not in nicotine-treated nAChR  $\alpha 7$  subunit knock-out ( $\alpha 7$  KO) mice. Moreover, nicotine-treated  $\alpha 7$  KO mice did not differ in disease response from that seen in  $\alpha 7$  KO or WT mice exposed instead to vehicle (Hao et al., 2011). The simplest interpretation was that elimination of  $\alpha 7^*$ -nAChR seemed to eliminate nicotine protection. However, the apparent absence of a difference in disease response in vehicle-treated  $\alpha 7$  KO compared to WT mice paradoxically suggested that  $\alpha 7^*$ -nAChR did not mediate natural cholinergic mechanisms involved in disease or in the response to disease (Hao et al., 2011). In addition, deeper characterization involved determinations of immune cell levels in the periphery and in the central nervous system (CNS) and of levels of expression of markers of inflammation and hyper-immunity. These levels were not statistically different for vehicle-treated  $\alpha 7$  KO or WT animals, again suggesting lack of involvement of  $\alpha 7^*$ -nAChR in natural, disease-related, cholinergic processes, which thus must involve other nAChR subtypes. Nevertheless, inflammatory and immune markers actually were suppressed in nicotine-treated  $\alpha 7$  KO animals, although not as much as by nicotine exposure in WT mice (Hao et al., 2011). Among other things, this suggested that nAChR subtypes in addition to  $\alpha 7^*$ -nAChR also must be involved in nicotine’s protective effects (Hao et al., 2011).

This intuition led to our finding that many immune system cell types express a number of nAChR subunits, most prominently  $\alpha 9$  and  $\beta 2$  subunits (Hao et al., 2011),

and that nicotine's antagonism of  $\alpha 9^*$ -nAChR appears to be responsible in large part for nicotine's protective effects, which are mimicked in nAChR  $\alpha 9$  subunit KO mice (Simard et al., 2013). This extended our knowledge about  $\alpha 9$  subunits and nAChR containing them, previously realized to be expressed in the cochlea, olfactory epithelium and skin, but not in the CNS (Jensen et al., 2005; Lukas and Bencherif, 2006; Taly et al., 2009). Warranting emphasis, and possibly because  $\alpha 9$  subunits are the most ancient of known, mammalian nAChR subunits,  $\alpha 9^*$ -nAChR have a unique and perhaps similarly ancient pharmacological profile, in some ways akin to GABA and glycine receptors, and respond to nicotine as though it were an antagonist, rather than an agonist, whereas nicotine is an agonist mimicking effects of ACh at other nAChR subtypes. Thus, the finding is to be expected that exposure to nicotine acting as an antagonist to block  $\alpha 9^*$ -nAChR function, or elimination of  $\alpha 9^*$ -nAChR in a nAChR  $\alpha 9$  subunit KO ( $\alpha 9$  KO) mouse, leads to the same level of disease attenuation in the EAE model. In this case, elimination of  $\alpha 9^*$ -nAChR eliminated nicotine protection, but this is because nicotine effectively already produces elimination of  $\alpha 9^*$ -nAChR. Importantly, the findings indicate that  $\alpha 9^*$ -nAChR naturally contribute to disease-exacerbating effects (Simard et al., 2013).

However, we require an improved understanding of roles played by  $\alpha 9^*$ -nAChR in anti- or pro-inflammatory responses, in disease-exacerbating or disease-attenuating immune or immunosuppressive responses, and in effects on those responses of nicotine exposure. Moreover, our understanding is deficient about what immune or other cell types express disease-relevant  $\alpha 9^*$ -nAChR. In addition, the same questions can be raised about roles played by  $\alpha 7^*$ -nAChR. For example, left open is the question about if and how  $\alpha 7^*$ - and  $\alpha 9^*$ -nAChR interact in modulation of inflammatory and immune processes. To address these issues, we leveraged the availability of nAChR  $\alpha 7/\alpha 9$  subunit double knock-out (DKO) mice for study, also using direct and adoptive transfer EAE models. The results confirm disease-exacerbating roles for  $\alpha 9^*$ -nAChR, apparently restricted to their expression in the periphery on immune system cells, but also reveal protective roles of  $\alpha 7^*$ -nAChR expressed centrally.

## MATERIALS AND METHODS

### Mice

C57BL/6J mice used as WT controls sometimes were purchased from Taconic Biosciences (Hudson, NY, USA), but they more typically were WT littermates or colony controls generated in the course of obtaining KO or DKO mice. Importantly, no differences were observed in any experiments between commercially-obtained C57BL/6 mice and the WT colony controls or littermates of the extensively backcrossed DKO mice used. The nAChR  $\alpha 9$  subunit KO mouse model was generated by the deletion of exons 1 and 2 and their flanking intronic sequences (Genoway, Inc., Lyon, France). The embryonic stem

cells used to make the transgenic mouse line were from the 129/svPas (129S2; 129/sv) strain. The  $\alpha 9$  KO line was backcrossed at the Boys Town National Research Hospital (Omaha, NB, USA) to C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) using Marker-Assisted Accelerated Backcrossing (MAX BAX; Charles River, Troy, NY, USA) until congenicity (99+%) was achieved. Details about the development of the  $\alpha 9$  KO line have been described elsewhere (Morley et al., 2017a,b). The animals are periodically backcrossed to C57BL/6J mice to prevent genetic drift of the background strain. The absence of  $\alpha 9$  transcript and protein in cells dissected from the retinas and inner ears of  $\alpha 9$  KO mice has been previously reported (Smith et al., 2014; Morley et al., 2017b).

The nAChR  $\alpha 7$  subunit KO strain was established from mice obtained from the Jackson Laboratories (JAX; RRID: IMSR\_JAX:000664)<sup>1</sup> as heterozygote breeders following eight generations of backcrossing at JAX. The animals were then further backcrossed for four generations at Boys Town National Research Hospital using MAX BAX, and they have since been periodically backcrossed with JAX C57BL/6J WT and nAChR  $\alpha 7$  subunit KO animals to prevent genetic drift.

The  $\alpha 7/\alpha 9$  DKO strain was established by breeding nAChR  $\alpha 9$  subunit KO heterozygote females with nAChR  $\alpha 7$  subunit KO males. Subsequent crossings used  $\alpha 7$  or  $\alpha 9$  female heterozygotes or  $\alpha 9$  KO females mated with  $\alpha 7$  or  $\alpha 9$  male heterozygotes or KO animals.

For the studies reported here, mice were bred and housed at Boys Town National Research Hospital until maturity and shipped by overnight courier to the Barrow Neurological Institute (Phoenix, AZ, USA). The animals were bred under presumed identical conditions and tested negative for all standard viruses and parasites by IDEXX (Columbia, MO, USA).

The litter sizes for all strains varied from 5 to 9. Dams and males in our colony are typically first bred at 6–8 weeks of age, not utilized for more than two litters, and are rarely bred past the age of 12–14 weeks. Young dams and males are used for breeding to reduce the incidence of epigenetic factors that might affect the phenotype.

All animal studies were approved by Animal Care and Use Committees of the Barrow Neurological Institute or Boys Town National Research Hospital.

### Immunostaining

T (CD4<sup>+</sup> or CD8<sup>+</sup>), monocyte/macrophage (CD11b<sup>+</sup>) or dendritic (CD11c<sup>+</sup>) cells from the spleens of WT or DKO mice were isolated using fluorescence-activated cell sorting (FACS) as described previously (Li et al., 2017). For FACS analysis, single cell suspensions (10<sup>6</sup> cells) of splenocytes were stained with fluorochrome-conjugated antibodies. Targets and antibodies used (BD Biosciences, Franklin Lakes, NJ, USA) were: CD3 (145-2C11), CD4 (GK1.4), CD8 (53–6.7), CD11b (M1/70), CD11c (HL3). Flow cytometric data were collected on a FACS Aria flow cytometer (BD Biosciences) and analyzed with Diva software. Isotype-matched negative control mAbs

<sup>1</sup><https://www.jax.org>



were used for all stains. Sorted cells were collected in conical tubes and then seeded on cover slips. For immunostaining, FACS-sorted cells were fixed in 4% paraformaldehyde for 10 min and washed at room temperature with phosphate-buffered saline (PBS) three times for 5 min each. A PBS-based solution containing 5% blocking serum and 0.3% Triton X-100 was applied for 1 h. Cells were incubated at 4°C overnight with primary antibodies and for 1 h at room temperature with Alexa Fluor-conjugated secondary antibodies. Cells were then washed three times for 5 min each with PBS and prepared for fluorescence microscopy as previously described (Liu et al., 2009, 2013). The following primary antibodies were used: goat anti-nAChR  $\alpha 9$  antibody (E-17, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-CD4 antibody (H-370, Santa Cruz Biotechnology), rat anti-CD8 antibody (2.43, Santa Cruz Biotechnology), rabbit anti-CD11b antibody (Abcam, San Francisco, CA, USA) and rabbit anti-CD11c antibody (EP1347Y, Abcam).

## EAE Induction

The mouse peptide MOG<sub>35–55</sub> (myelin oligodendrocyte glycoprotein; amino acids MEVGWYRSPFSRVVHLYRNGK) was synthesized (purity >95%) by Bio-Synthesis, Inc. (Lewisville, TX, USA). To induce EAE directly, mice were injected sub-cutaneously in the hind flank with 200  $\mu$ g MOG<sub>35–55</sub> peptide in complete Freund's adjuvant (BD Biosciences) containing 500  $\mu$ g of nonviable desiccated *Mycobacterium tuberculosis*. On the day of immunization and 2 days after, mice also were injected intravenously with 200 ng pertussis toxin (List Biological Laboratories, Campbell, CA, USA). Passive induction (adoptive transfer) EAE was initiated by transfers of splenocytes isolated from donor mice in which a direct EAE response was initiated as previously described (Huang et al., 2006; Hao et al., 2010). In brief, splenocytes were obtained from either WT or  $\alpha 7/\alpha 9$  DKO donor mice, treated with either PBS vehicle or with nicotine starting on the day of immunization, 14 days after immunization with MOG<sub>35–55</sub>. Thereafter, splenocytes were cultured in the presence of 30  $\mu$ g/ml MOG<sub>35–55</sub> and 20 ng/ml recombinant interleukin-12 (R&D Systems). After culture for 3 days, cells were collected and washed in PBS.  $1 \times 10^7$  splenocytes were transferred into Rag2<sup>-/-</sup> mice by intravenous injection. Recipient mice also were given 200 ng pertussis toxin intravenously on the day of cell transfer and 2 days after transfer. Whether for direct or adoptively transferred EAE, mice or recipient mice were monitored daily for symptoms scored on an arbitrary scale of 0–5 as previously described (Hao et al., 2011; Simard et al., 2013).

## Histological Analyses

At specific times after direct or adoptively transferred EAE induction, mice displaying disease scores typical of their group were anesthetized by 2% isoflurane inhalation and perfused by intracardiac puncture with 50 ml of cold PBS. Spinal cords were removed and fixed in 10% formalin/PBS. Paraffin-embedded, longitudinal sections running across the cervical enlargement were prepared and stained for infiltrating immune

cells (hematoxylin and eosin), myelin (luxol fast blue) and axons (Biechowky silver). Manual tracing was used to define the degree of inflammation, demyelination, and axonal damage across the entire spinal cord section for each mouse. Evidence of pathology was scored as follows: 0, no changes; 1, focal area involvement; 2, <5% of total myelin area involved; 3, 5%–10% of total myelin area involved; 4, 10%–20% involved area; 5, >20% of total myelin area involved (Bai et al., 2004).

## Nicotine Treatment

(–)Nicotine bitartrate was purchased from Sigma (St. Louis, MO, USA). A 100 mg/ml solution of nicotine salt in PBS, or a solution containing PBS alone, were freshly prepared and loaded into Alzet<sup>®</sup> osmotic minipumps (model 1004, Durect Corporation, Cupertino, CA, USA) 24 h before pump implantation as described (Simard et al., 2013). On the day of MOG immunization, the minipumps were implanted subcutaneously on the right side of the back of the mouse and continuously delivered either PBS or nicotine salt at 12  $\mu$ l/d until the end of experiments. This dosing regimen is designed to produce plasma levels of nicotine in the relevant animals of  $\sim 49$  ng/ml or  $\sim 300$  nM, which is comparable to concentrations of nicotine typically found in human cigarette smokers and is behaviorally relevant in mice (Matta et al., 2007).

## 7T Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) was performed using a 7 Tesla, small animal, 30 cm horizontal-bore magnet and BioSpec Avance III spectrometer (Bruker Biospin MRI, Billerica, MA, USA) as previously described (Hao et al., 2010; Liu et al., 2017). Mice were under anesthesia by inhalation of 3.5% isoflurane and maintained by inhalation of 1%–2% isoflurane in 70% N<sub>2</sub>O and 30% O<sub>2</sub> delivered via a face mask. During MRI scans, animal respiration was continually assessed by a small animal monitoring and gating system (SA Instruments, Stony Brook, NY, USA) via a pillow sensor positioned under the abdomen. Axial 2D multi-slice T2-weighted images of the brain were acquired with fat-suppressed Rapid Acquisition with Relaxation Enhancement sequence (TR = 4000 ms, effective TE = 60 ms, number of average = 4, FOV = 19.2 mm  $\times$  19.2 mm, matrix size = 192 mm  $\times$  192 mm, slice thickness = 0.5 mm, TA = 6 m 24 s). MRI data were analyzed using the MEDx3.4.3 software package (Medical Numerics, Germantown, MD, USA) on a LINUX workstation.

## In Vivo CNS Bioluminescence

To assess reactive oxygen species (ROS) production in brain, bioluminescence images were captured in live mice using a Xenogen IVIS200 imager (Caliper Life Sciences, Hopkinton, MA, USA) 20 min after i.p. injection of 100  $\mu$ l of 50 mg/ml Luminol (Sigma-Aldrich, St. Louis, MO, USA) as we previously described (Hao et al., 2010; Simard et al., 2013). Regions of interest were selected to measure luminescence intensity in the brain. Data were collected as photons/sec/cm<sup>2</sup> using Living Image<sup>®</sup> software (Caliper Life Sciences, Hopkinton, MA, USA).

## Statistical Analyses

Data are presented as Mean  $\pm$  SEM. Differences were considered significant at  $p < 0.05$ . Statistical differences among groups were evaluated by two-tailed unpaired Student's *t*-test for two groups and by one-way analysis of variance (ANOVA) followed by Tukey *post hoc* test for three or more groups. Two-way ANOVA accompanied by a Bonferroni *post hoc* test was used for multiple comparisons. All statistical analyses were performed using Prism 5.0 software (GraphPad, San Diego, CA, USA).

## RESULTS

### Expression Profile of nAChR $\alpha 9$ Subunit in Immune Cells

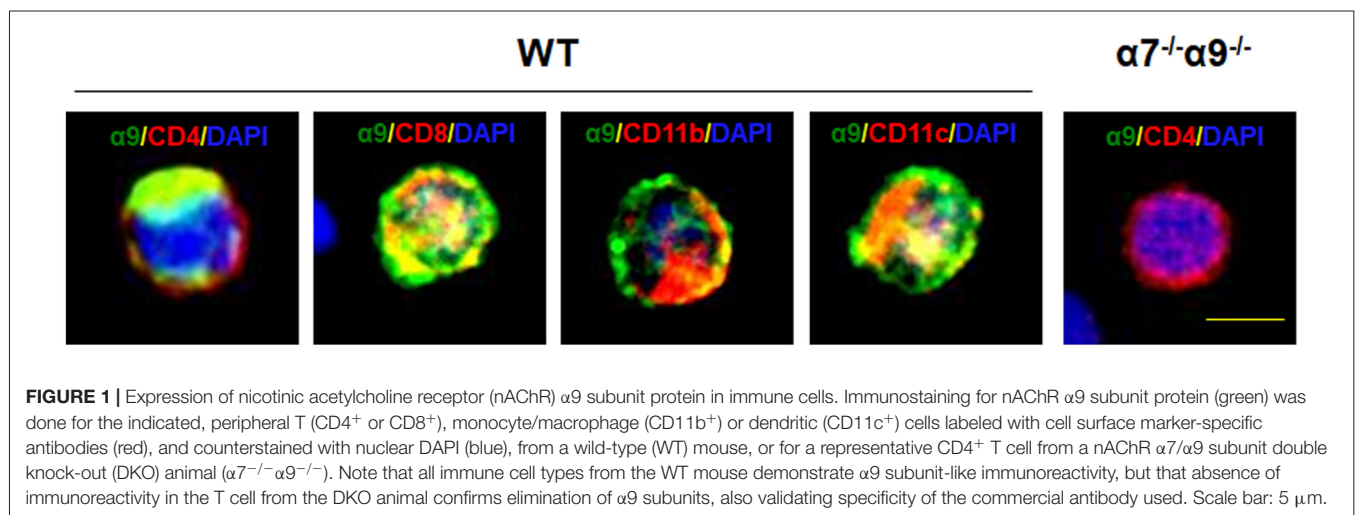
To validate the expression of the nAChR  $\alpha 9$  subunit gene as protein in selected immune cell types, we subjected T (CD4<sup>+</sup> or CD8<sup>+</sup>), monocyte/macrophage (CD11b<sup>+</sup>) or dendritic (CD11c<sup>+</sup>) cells, isolated by FACS from the spleens of WT mice, to immunostaining with cell-specific markers and with an antibody against  $\alpha 9$  subunits. All of these immune cell types display nAChR  $\alpha 9$  subunit-like immunoreactivity (**Figure 1**). However, similar assessment in immune cells from nAChR  $\alpha 9$  subunit KO mice were negative for subunit immunoreactivity (results for CD4<sup>+</sup> T cells are shown; **Figure 1**). Interestingly, CD4<sup>+</sup> T cells from nAChR  $\alpha 9$  subunit KO mice are smaller than those from WT mice, likely indicative of immaturity.

### Nicotine Treatment Attenuates Direct EAE Severity in Both WT and $\alpha 7/\alpha 9$ DKO Mice, but in the Absence of Nicotine Treatment, There Is Exacerbation of Direct EAE Severity in DKO Compared to WT Animals

Our previous studies initially indicated equivalence, in disease scores and other indications of immunity and inflammation, between WT animals continuously exposed to nicotine and nAChR  $\alpha 9$  subunit KO animals irrespective of whether they were exposed to nicotine or vehicle. Each of these three

cohorts of animals had reduced severity or other indices of disease compared to vehicle-treated WT animals (Simard et al., 2013). Our initial studies using nAChR  $\alpha 7$  subunit KO animals suggested that they did not differ from WT animals in disease score measures when treated with vehicle alone, or when  $\alpha 7$  KO mice were exposed to nicotine, which was protective against disease in WT animals (Hao et al., 2011). Other markers of immunity and inflammation in vehicle-treated  $\alpha 7$  subunit KO animals or WT mice were indistinguishable. However, nicotine exposure did produce attenuation of indices of immunity and inflammation in  $\alpha 7$  KO animals, although not at the higher levels of attenuation seen in nicotine-treated WT mice. These findings suggested involvement of other nAChR subtypes in disease and protection against it. Although recognizing that there might be potential challenges in data interpretation in such studies, we nevertheless sought to determine effects on disease and nicotine protection in animals lacking both nAChR  $\alpha 7$  and  $\alpha 9$  subunits.

Comparable degrees of protection against disease in the direct EAE model again are seen in WT animals and in  $\alpha 7/\alpha 9$  DKO mice continuously exposed to nicotine (**Figure 2; Table 1**). These effects also are equivalent to those seen upon deletion only of  $\alpha 9$  subunits (Simard et al., 2013). Treatment with the ligand delays the onset (by 2 days in this cohort) and attenuates the severity of disease response (by  $\sim 25\%$ – $33\%$ ) relative to the disease course in control, vehicle-treated WT animals (peak clinical scores:  $2.8 \pm 0.2$  WT/vehicle vs.  $1.9 \pm 0.1$  WT/nicotine,  $p < 0.01$ ; vs.  $2.1 \pm 0.1$   $\alpha 7/\alpha 9$  DKO/nicotine,  $p < 0.01$ ; **Figure 2; Table 1**). However, and remarkably, direct EAE severity is elevated (by  $\sim 36\%$ ) in  $\alpha 7/\alpha 9$  DKO mice exposed only to vehicle relative to effects in WT control animals (peak clinical scores:  $2.8 \pm 0.8$ , WT/vehicle vs.  $3.8 \pm 0.8$   $\alpha 7/\alpha 9$  DKO/vehicle,  $p < 0.01$ ; **Figure 2; Table 1**; Disease severity:  $\alpha 7/\alpha 9$  DKO:saline  $>$  WT:saline  $>$   $\alpha 7/\alpha 9$  DKO:nicotine = WT:nicotine). Differences between groups in peak disease severity are preserved over the  $\sim 2$  weeks when recovery occurs in these animals.

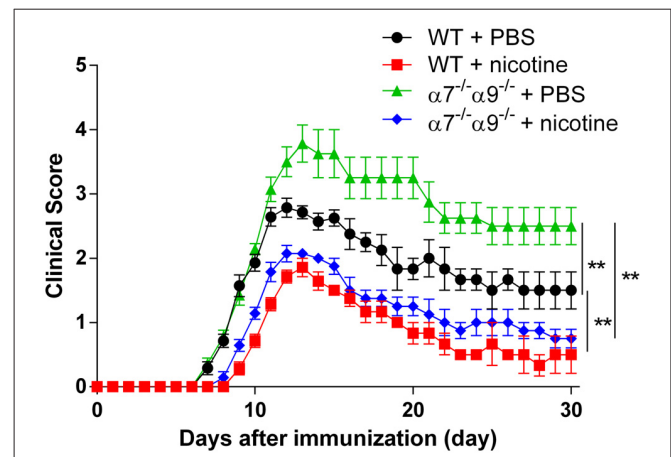


**FIGURE 1** | Expression of nicotinic acetylcholine receptor (nAChR)  $\alpha 9$  subunit protein in immune cells. Immunostaining for nAChR  $\alpha 9$  subunit protein (green) was done for the indicated, peripheral T (CD4<sup>+</sup> or CD8<sup>+</sup>), monocyte/macrophage (CD11b<sup>+</sup>) or dendritic (CD11c<sup>+</sup>) cells labeled with cell surface marker-specific antibodies (red), and counterstained with nuclear DAPI (blue), from a wild-type (WT) mouse, or for a representative CD4<sup>+</sup> T cell from a nAChR  $\alpha 7/\alpha 9$  subunit double knock-out (DKO) animal ( $\alpha 7^{-/-}\alpha 9^{-/-}$ ). Note that all immune cell types from the WT mouse demonstrate  $\alpha 9$  subunit-like immunoreactivity, but that absence of immunoreactivity in the T cell from the DKO animal confirms elimination of  $\alpha 9$  subunits, also validating specificity of the commercial antibody used. Scale bar: 5  $\mu$ m.

## Brain Lesion Volume Is Larger, and Levels of Reactive Oxygen Species Are Elevated, at Peak, Directly-Induced EAE, in $\alpha 7/\alpha 9$ DKO Mice Compared to Results using WT Animals, but Nicotine Treatment Lessens Lesion Volume and Inflammation in Both $\alpha 7/\alpha 9$ DKO and WT Mice

Consistent with disease scores, MRI assessments of brain lesion volume at disease peak are reduced (~18%–27%) in nicotine-treated, WT or  $\alpha 7/\alpha 9$  subunit DKO mice relative to effects seen in WT animals treated with vehicle (Figure 3). Lesion volumes are  $5.2 \pm 0.5 \text{ mm}^2$  for WT/vehicle mice vs.  $3.8 \pm 0.3 \text{ mm}^2$  for WT/nicotine animals ( $p < 0.01$ ) and  $4.3 \pm 0.5 \text{ mm}^2$  for  $\alpha 7/\alpha 9$  DKO/nicotine mice ( $p < 0.01$ ). However, compared to results in vehicle-treated WT mice, lesion volumes are elevated by about 37% in vehicle-treated  $\alpha 7/\alpha 9$  DKO mice ( $7.1 \pm 1.1 \text{ mm}^2$ ), also consistent with the higher disease scores in these animals (Brain lesion volume:  $\alpha 7/\alpha 9$  DKO:saline > WT:saline >  $\alpha 7/\alpha 9$  DKO:nicotine = WT:nicotine).

Similarly, bioluminescence measurements [quantified in (photons/sec/cm<sup>2</sup>)/10<sup>5</sup>] of brain inflammation at peak disease based on ROS visualization are ~16%–26% lower, when compared to those in vehicle-treated WT animals ( $4.3 \pm 0.3$ ), in both nicotine-treated WT mice ( $3.2 \pm 0.3$ ) or nicotine-treated  $\alpha 7/\alpha 9$  DKO animals ( $3.6 \pm 0.4$ ;  $p < 0.01$  for both; Figure 4). Nevertheless, there is significantly elevated (~21%) CNS ROS production in vehicle-treated  $\alpha 7/\alpha 9$  DKO ( $5.2 \pm 0.5$ ;  $p < 0.01$  to each of the other groups (Figure 4), again reflecting higher disease scores (and brain lesion volume) in the latter group (Figure 4; Brain inflammation:  $\alpha 7/\alpha 9$  DKO:saline > WT:saline >  $\alpha 7/\alpha 9$  DKO:nicotine = WT:nicotine).



**FIGURE 2 |** Exacerbated, direct experimental autoimmune encephalomyelitis (EAE) severity in  $\alpha 7^{-/-}\alpha 9^{-/-}$  mice. EAE disease symptom evaluation was done for groups of WT or nAChR  $\alpha 7/\alpha 9$  subunit DKO ( $\alpha 7^{-/-}\alpha 9^{-/-}$ ) mice receiving phosphate-buffered saline (PBS) or nicotine treatment. PBS or nicotine was delivered via minipumps until the end of experiment. The minipumps were implanted subcutaneously and continuously delivered either PBS or nicotine salt at an equivalent of ~13 mg of nicotine free base/kg/day. In the absence of nicotine treatment, exacerbated EAE severity was seen in  $\alpha 7^{-/-}\alpha 9^{-/-}$  mice ( $\alpha 7^{-/-}\alpha 9^{-/-}$  + PBS; ▲;  $n = 8$ ) vs. WT controls (WT + PBS; ●;  $n = 7$ ). Nicotine treatment reduced EAE severity in both  $\alpha 7^{-/-}\alpha 9^{-/-}$  ( $\alpha 7^{-/-}\alpha 9^{-/-}$  + nicotine; ◆;  $n = 7$ ) and WT mice (WT + nicotine; ■;  $n = 7$ ). Mean  $\pm$  SEM; two-way analysis of variance (ANOVA); \*\* $p < 0.01$ .

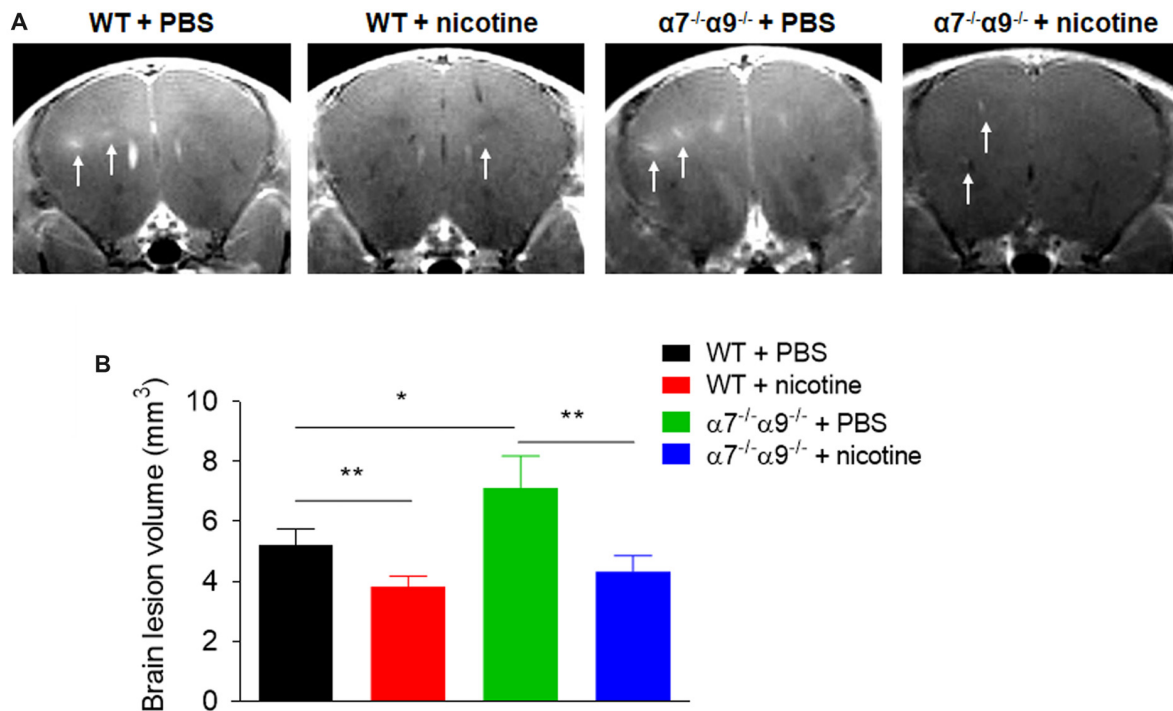
## Protection Against Adoptively Transferred EAE Induced by Nicotine Exposure or by Deletion of Both nAChR $\alpha 7$ and $\alpha 9$ Subunits in Donors

$\alpha 7^*$ -nAChR are widely dispersed in central and autonomic nervous systems and are present in immune cell types.

**TABLE 1 |** Summary of EAE studies across nAChR subunit genotypes and treatments.

Animal (genetic status)	Treatment	Direct EAE severity (+, ++, +++ scale)	Reference(s)	Adoptive transfer EAE severity (genetic status and treatment of donor; wild-type recipient; +, ++, +++ scale)	Reference(s)
Wild-type	Saline	++	Shi et al. (2009); Hao et al. (2011); Simard et al. (2013)	++	Shi et al. (2009) This report <b>Figure 3</b>
	Nicotine	+	This report <b>Figure 1</b>	+	
$\alpha 7$ KO	Saline	++	Hao et al. (2011)		
	Nicotine	++			
$\alpha 9$ KO	Saline	+	Simard et al. (2013)		
	Nicotine	+			
$\alpha 7/\alpha 9$ DKO	Saline	+++	This report <b>Figure 1</b>	+	This report <b>Figure 3</b>
	Nicotine	+		+	

Direct or adoptive EAE severity scores (scale of + to +++) are illustrated for animals with the indicated genotype and treatments (nicotine to yield blood levels of ~300 nM or saline alone) following experimental procedures as described in the "Materials and Methods" Section of this report or in the cited publication(s). Disease severity in direct EAE (++ level) is like that in wild-type (WT) animals treated with saline or in nAChR  $\alpha 7$  subunit knock-out ( $\alpha 7$  KO) animals irrespective of nicotine or saline treatment (WT:saline =  $\alpha 7$  KO:saline =  $\alpha 7$  KO:nicotine) and also is at positive control levels of disease in recipients of adoptively transferred splenocytes from WT animals treated with saline. Direct EAE disease severity is attenuated to essentially equivalent degrees in WT,  $\alpha 9$  KO, or  $\alpha 7/\alpha 9$  double knock-out (DKO) animals treated with nicotine, or in  $\alpha 9$  KO animals treated with saline (WT:nicotine =  $\alpha 9$  KO:nicotine =  $\alpha 7/\alpha 9$  DKO:nicotine =  $\alpha 9$  KO:saline), and there is similar attenuation of adoptively transferred EAE disease severity upon nicotine treatment of WT or  $\alpha 7/\alpha 9$  DKO donor animals or for saline-treated  $\alpha 7/\alpha 9$  DKO mice. Remarkably, direct EAE disease severity is worst for saline-treated  $\alpha 7/\alpha 9$  DKO mice.



**FIGURE 3 |** Increased brain lesion size in  $\alpha 7^{-/-}\alpha 9^{-/-}$  mice during the peak phase of direct EAE. **(A)** T1-weighted images were obtained with a 7T magnetic resonance imaging (MRI) scanner in WT or nAChR  $\alpha 7/\alpha 9$  subunit DKO ( $\alpha 7^{-/-}\alpha 9^{-/-}$ ) mice receiving PBS or nicotine treatment. Mice received i.v. injections of gadolinium prior to MRI scans. Arrows indicate focal lesions in the brain and increased signal intensity on T1-weighted images. MRI scans were conducted in groups of mice ( $n = 5$ ) at day 14 after immunization. PBS or nicotine was delivered via minipumps until the end of experiment. The minipumps were implanted subcutaneously and continuously delivered either PBS or nicotine salt at an equivalent of  $\sim 13$  mg of nicotine free base/kg/day. **(B)** Bar graph shows quantified data. Mean  $\pm$  SEM; one-way ANOVA; \* $p < 0.05$ , \*\* $p < 0.01$ .

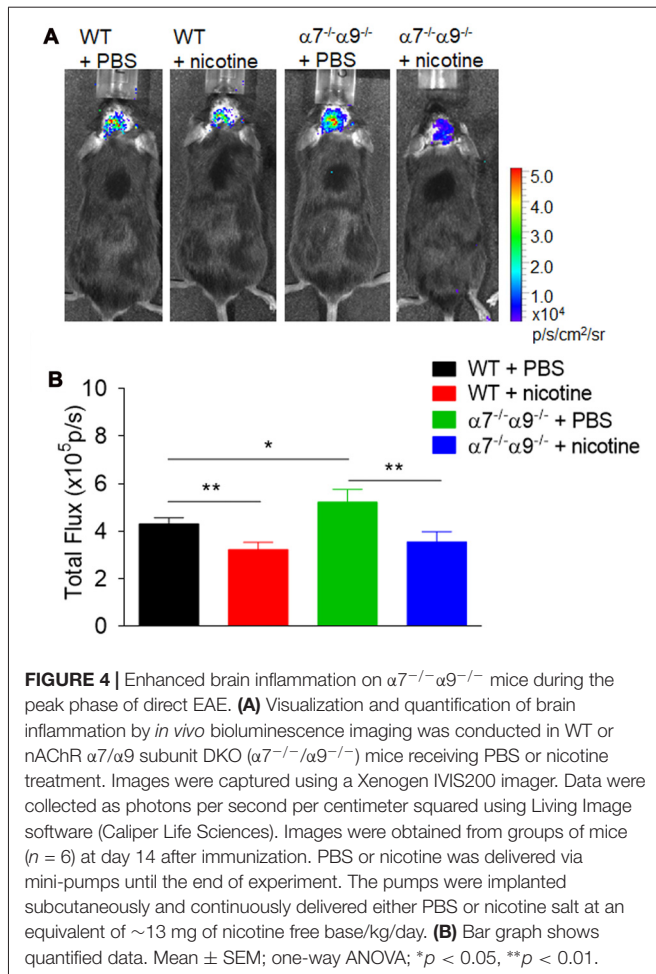
$\alpha 9^*$ -nAChR appear to be absent in the CNS but are present much more abundantly than  $\alpha 7^*$ -nAChR in immune system cells (see “Discussion” Section). To determine effects of selective elimination of both subunits from immune system cells capable alone of conferring EAE to recipient mice, we also established effects of nAChR  $\alpha 7/\alpha 9$  DKO in the adoptive transfer model of EAE. WT or DKO donor mice were treated with vehicle alone or supplemented with nicotine and inoculated with MOG peptide. Total splenocytes taken from animals at peak disease were isolated and transferred in Rag2<sup>-/-</sup> recipients having the full contingent of nAChR subunits but lacking T, natural killer T, and B cells. Rag2<sup>-/-</sup> recipients receiving splenocytes from vehicle-treated WT animals develop severe EAE (peak clinical score  $3.5 \pm 0.2$ ; **Figure 5; Table 1**). However, EAE disease symptoms are attenuated ( $\sim 30\%$ ) and delayed ( $\sim 2$ – $3$  days) in recipients of splenocytes from nicotine-treated WT mice ( $2.2 \pm 0.1$ ) or from nicotine-treated DKO animals ( $2.4 \pm 0.2$ ;  $p < 0.01$  for both; **Figure 5; Table 1**). A comparable degree of protection against adoptively transferred EAE is also seen for Rag2<sup>-/-</sup> mice receiving splenocytes from DKO animals treated with vehicle alone (peak clinical score of  $2.6 \pm 0.2$ ;  $p < 0.01$  relative to WT/vehicle control; **Figure 5; Table 1**; Disease severity: WT:saline >  $\alpha 7/\alpha 9$  DKO:saline =  $\alpha 7/\alpha 9$  DKO:nicotine = WT:nicotine).

## DISCUSSION

### Immunostaining with a Validated Antibody Confirms nAChR $\alpha 9$ Subunit Expression as Protein in Immune System Cells, and T Cell Maturation Appears to be Inhibited in nAChR $\alpha 9$ Subunit KO Mice

Results in this study indicate that many immune cell types express nAChR  $\alpha 9$  subunits not only as mRNA, as previously shown (Hao et al., 2011), but also as protein, identified by immunostaining of peripheral (splenic) CD4<sup>+</sup> and CD8<sup>+</sup> T cells, CD11b<sup>+</sup> monocytes/macrophages, and CD11c<sup>+</sup> dendritic cells. This is consistent with other results demonstrating function of peripheral immune cell  $\alpha 9^*$ -nAChR in disease model studies based on gene deletion or nicotine antagonism (Simard et al., 2013). The finding that immunostaining is absent in nAChR  $\alpha 7/\alpha 9$  DKO mice lacking  $\alpha 9$  subunits supports specificity of the anti- $\alpha 9$  subunit antibody used, which is important to demonstrate for anti-nAChR subunits (Jones and Wonnacott, 2005; Moser et al., 2007). Reciprocally, that observation also demonstrates that genetic elimination of  $\alpha 9$  subunits translates into an absence of subunit protein. The finding that CD4<sup>+</sup> T cell size is smaller in  $\alpha 9$  KO animals is consistent with earlier studies showing that nicotine

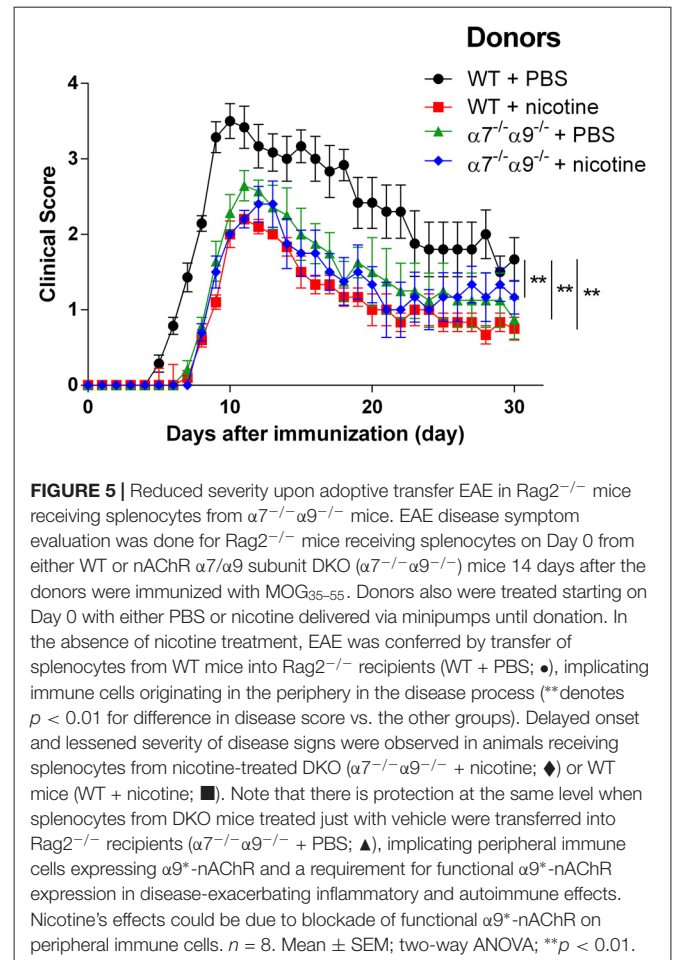




exposure, which antagonizes  $\alpha 9^*$ -nAChR function, inhibits T cell differentiation and maturation, manifest as smaller size of those cells produced in thymic organ cultures and when assessed using flow cytometry (Middlebrook et al., 2000, 2002). The observation that elimination of nAChR  $\alpha 9$  subunits (and  $\alpha 9^*$ -nAChR) has anti-inflammatory and immunosuppressive effects is consistent with evident immaturity or anergy of T cells in  $\alpha 9$  KO animals.

### $\alpha 9^*$ -nAChR Roles in Disease Exacerbating Effects

In the direct EAE model, nicotine exposure is confirmed again to delay the onset and to attenuate the severity of EAE in WT mice, and effects are shown here for the first time to be similar in nicotine-treated  $\alpha 7/\alpha 9$  DKO animals. Brain lesion volumes and levels of ROS markers of inflammation are comparably lowered in nicotine-treated WT or  $\alpha 7/\alpha 9$  DKO mice, paralleling as expected effects on the integrated response measured by disease score. This all is consistent with the equivalence of nicotine's protective effects in WT or  $\alpha 9$  KO mice, mimicking effects in vehicle-treated  $\alpha 9$  KO mice; nicotine's protective effects wouldn't be expected to add to already protective effects of nAChR  $\alpha 9$  subunit



deletion in  $\alpha 9$  KO mice (as seen in Simard et al., 2013). The interpretation of this collective body of evidence is that  $\alpha 9^*$ -nAChR play roles in initiation or exacerbation of disease-relevant inflammatory and immune responses. Their antagonism by nicotine or their elimination in  $\alpha 9$  KO mice lessens disease burden and disease-exacerbating inflammatory and immune responses.

### Multiple Targets and Disease Thresholds for Nicotine's Protective Effects

In the directly-induced EAE model, protective effects of nicotine exposure in WT and in  $\alpha 7/\alpha 9$  DKO mice contrast with the initially-apparent loss of nicotine's protective effects, at least in terms of the integrated EAE disease response, in nAChR  $\alpha 7$  KO mice (Hao et al., 2011). Why would sustained nicotine exposure not appear to be protective in  $\alpha 7$  KO mice but protective in  $\alpha 7/\alpha 9$  DKO mice, where its antagonism of  $\alpha 9^*$ -nAChR would be expected to be redundant with  $\alpha 9^*$ -nAChR elimination? One possibility is that the integrated disease response emerges once a threshold of inflammatory activity and hyper-immunity is achieved. That would imply that nicotine treatment in  $\alpha 7$  KO mice inadequately suppresses such activity to below threshold levels. This is consistent with observations that nicotine treatment does diminish other indices of inflammation and

immunity in  $\alpha 7$  KO mice, but just not to the same degree as nicotine attenuates such responses in WT animals (Hao et al., 2011). A related interpretation is that  $\alpha 7^*$ -nAChR play protective roles, and that their elimination in  $\alpha 7$  KO animals lowers the threshold for development of disease to a level that is not overcome by nicotine exposure. Our previous studies have shown that expression levels of cytokines or other inflammatory factors change predictably in reflection of disease severity, but inflammatory status of  $\alpha 7/\alpha 9$  DKO mice during EAE warrants further investigation, as we plan. Regardless, it is evident from nicotine's protective roles in  $\alpha 7/\alpha 9$  DKO mice that nicotine has additional targets other than  $\alpha 9^*$ - and  $\alpha 7^*$ -nAChR that are relevant to the EAE inflammatory and immune process (Simard et al., 2013).

### Newly Revealed, Natural Protective Effects of $\alpha 7^*$ -nAChR

Another important observation is that directly-induced EAE severity is elevated in vehicle-treated  $\alpha 7/\alpha 9$  DKO mice compared to vehicle-treated WT animals. This contrasts with the aforementioned protective effects of  $\alpha 9$  KO alone and with the initially-apparent null effect of  $\alpha 7$  KO alone when disease manifestation is considered in comparison to that in vehicle-treated WT animals. Viewed one way, our interpretation is that there is a loss of protection against direct EAE due to elimination of naturally-functioning  $\alpha 7^*$ -nAChR, even when  $\alpha 9^*$ -nAChR also are deleted. Phrased differently, we postulate that upon elimination of disease-exacerbation attributable to  $\alpha 9^*$ -nAChR expression, additional elimination of  $\alpha 7$  subunits reveals protective effects of  $\alpha 7^*$ -nAChR responding to natural chemical signals.

### Importance in Disease Exacerbation of Peripheral Immune Cell $\alpha 9^*$ -nAChR, and Support for Protective Roles of $\alpha 7^*$ -nAChR in the CNS

The interpretations and hypotheses advanced here thus far were tested in adoptive transfer EAE studies. Our previous work showed that nicotine treatment is comparably if not more potent in attenuating disease signs in the passive EAE model employing transfer of immune system cells from inoculated, nicotine- or vehicle-treated, WT donors to otherwise naïve, Rag2<sup>-/-</sup> recipients when compared to nicotine's protective effects against direct EAE (Shi et al., 2009). Here we show that nicotine exposure in inoculated, WT or  $\alpha 7/\alpha 9$  DKO animals indeed protects against disease manifestation in Rag2<sup>-/-</sup> recipients of immune cells from those donors compared to effects seen for transfer of immune cells from vehicle-treated WT animals. Moreover, adoptive transfer of cells from vehicle-treated  $\alpha 7/\alpha 9$  DKO animals similarly produces attenuated disease in recipients. Our interpretation of these observations is that nicotine antagonism of  $\alpha 9^*$ -nAChR, or genetic deletion of  $\alpha 9^*$ -nAChR, has protective effects because they block or eliminate functional  $\alpha 9^*$ -nAChR in peripheral immune cells. This finding makes sense given the known disposition of  $\alpha 9^*$ -nAChR (Elgoyhen et al., 1994; Jensen et al., 2005). It is reported

that nAChR  $\alpha 9$ -like immunoreactivity seems to be present in the healthy piglet or infant mouse brain (Vivekanandarajah et al., 2015, 2016). However, previous *in situ* hybridization or other measures of transcript levels (e.g., see Elgoyhen et al., 1994; Hao et al., 2011), validated by recent RefSeq data<sup>2</sup>, indicate that healthy mouse brain expression of nAChR  $\alpha 9$  subunit mRNA is vanishingly low (below or at detection levels), in contrast to robust thymic (e.g., 11.423 RPKM) or immune cell levels. Furthermore, our own observations (in preparation) are entirely consistent with RefSeq<sup>3</sup> data indicating that healthy human CNS levels of nAChR  $\alpha 9$  subunits also are vanishingly low ( $0.154 \pm 0.104$  RPKM), whereas immune cell levels are substantial. Further work will ascertain whether these situations are different in diseased brain or immune system cells, across species, or across different techniques for assessment of subunit protein or message levels.

In addition, these findings indicate that elimination of nAChR  $\alpha 9$  subunits in immune cells is protective even in the absence of immune cell  $\alpha 7$  subunits and  $\alpha 7^*$ -nAChR. Moreover, the fact that adoptively-transferred disease is attenuated in the passive EAE model rather than exacerbated, as seen in the direct EAE model, in vehicle-treated  $\alpha 7/\alpha 9$  DKO mice, indicates that elimination of  $\alpha 7^*$ -nAChR in peripheral immune cells is not damaging. This finding also supports the hypothesis/interpretation that protective roles for  $\alpha 7^*$ -nAChR revealed in direct EAE studies using  $\alpha 7/\alpha 9$  DKO mice are due to actions of those  $\alpha 7^*$ -nAChR in the CNS.

### Past, Present and Future Perspectives

Delicate balancing of pro- vs. anti-inflammatory responses, and between activation or suppression of immune responses, requires cross-talk between the immune and nervous systems. Such cross-talk, and neuroimmune sharing of signaling molecules and mechanisms, even contributes to nervous system development and plasticity (Boulanger, 2009; Shatz, 2009; Besedovsky and del Rey, 2011). The chemical signaling agent, ACh, now is realized not just to have specialized activity as a neurotransmitter in the nervous system, but also to have actions in non-neuronal tissues and organs, such as in the immune system (Grando et al., 2012). There is no doubt that chemical sensing existed long before the emergence of nervous systems and concurrent specialization of chemical messengers and their targets to execute synaptic neurotransmission (Wessler and Kirkpatrick, 2008). ACh is among the primordial chemical messengers involved in chemical sensing long before they acquired more specialized functions as synaptic vesicle-packaged neurotransmitters. Even in advanced organisms with robust nervous systems, there now is solid evidence for cholinergic signaling and for the existence of enzymes that synthesize and degrade ACh in other organs and cell types, including in the immune system (Sato et al., 1999; Matsunaga et al., 2001; Kuo et al., 2002; Middlebrook et al., 2002; Kawashima et al., 2007; Fujii et al., 2008; Koval et al., 2011). Implications are that locally-synthesized and released ACh plays paracrine- or autocrine-type roles in these organs, but

<sup>2</sup><https://www.ncbi.nlm.nih.gov/gene/231252>

<sup>3</sup><https://www.ncbi.nlm.nih.gov/gene/55584>

not discounted are possible origins of ACh from terminals of neurons providing fine innervation of those organs. However, our understanding is immature about how this “non-neuronal” cholinergic signaling is leveraged and the depth and breadth of its involvement in control of extra-neuronal cell and organ function.

$\alpha 7^*$ -nAChR were the first suggested to be involved in the so-called “cholinergic anti-inflammatory pathway” (Wang et al., 2003; Shytle et al., 2004). That implication now indeed seems validated, despite the initially-observed null effect of  $\alpha 7$  KO on direct EAE disease signs (Nizri et al., 2009; Hao et al., 2011) which suggested that  $\alpha 7$ -nAChRs are not key players in endogenous, nAChR-dependent, immunoregulatory processes in that model. Instead, the exacerbation of direct EAE disease in  $\alpha 7/\alpha 9$  DKO mice reveals protective roles of  $\alpha 7^*$ -nAChR responding to natural cholinergic signals. This also is consistent with deeper analysis of the results using  $\alpha 7$  KO animals showing some, but only partial, attenuation of other inflammatory and immune response indices in the presence of nicotine as opposed to vehicle, thus revealing that  $\alpha 7^*$ -nAChR mediate some but not all of the effects of nicotine treatment, as one might expect for nAChR also mediating natural cholinergic signals (Hao et al., 2011). However, such roles do not seem to be due to  $\alpha 7^*$ -nAChR in peripheral immune cells, based on adoptive transfer studies using  $\alpha 7/\alpha 9$  DKO mice. Nevertheless, nicotine protection remains evident in direct EAE studies using  $\alpha 7/\alpha 9$  DKO mice, suggesting that additional nAChR subtypes are involved, not only in its effects, but also in natural cholinergic mechanisms.

In interpretation of effects of sustained nicotine exposure, there needs to be cognizance of how those effects can deviate substantially from those of acute exposure to nicotine (Lukas, 1991, 1995, 1998; Lukas et al., 1996; Gentry and Lukas, 2002; Gentry et al., 2003). Acute exposure to nicotine or ACh stimulates nAChR channel opening (as noted above, except for  $\alpha 9^*$ -nAChR, where nicotine is an antagonist rather than an agonist like ACh), but this effect is only transient. More sustained exposure can lead to functional “desensitization,” and then to progressively “deeper” levels of “functional”/“persistent” inactivation (Lukas et al., 1996; Gentry and Lukas, 2002). These longer-term effects, just as for acute actions of ligands, differ across nAChR subtypes with regard to potency, and they likely reflect a mixture of receptor activation and inactivation (Picciotto et al., 2008). It remains controversial whether nAChR subtypes, such as  $\alpha 7^*$ -nAChR, would be affected at all by nicotine at concentrations achieved via minipump delivery as used in the current studies. It also is not entirely clear whether nicotine exposure as produced in our studies would promote low level activation of specific nAChR subtypes or their functional inactivation. It also is possible that nAChR channel function is not relevant in the immune cell context, where nAChR occupancy might be adequate to alter intracellular signaling. Even for  $\alpha 9^*$ -nAChR, it is not clear that nicotine exposure as in these studies would be sufficient to antagonize channel function, especially if overcome by natural cholinergic signals. Also to be defined is where and how immune cells are exposed to natural cholinergic signals. Thus, much more work is required to define mechanisms involved in the clear and indisputable

protection against disease in the EAE model mediated by nicotine.

Evidence continues to build that phylogenetically-ancient  $\alpha 9^*$ -nAChR naturally play disease-exacerbating roles coincident with elevated immunity and inflammation. This challenges the notion of the cholinergic anti-inflammatory system, because  $\alpha 9^*$ -nAChR function seems to be anything but anti-inflammatory. Indeed, roles of  $\alpha 9^*$ -nAChR in endogenous pro-inflammatory mechanisms contributing to disease initiation and evolution are likely triggered by the endogenous agonist, ACh. Yet to be tested are hypotheses that function of  $\alpha 9^*$ -nAChR is required or important for maturation of T cells and perhaps other immune cell types. A consequence of nicotine antagonism-mediated  $\alpha 9^*$ -nAChR functional blockade or of  $\alpha 9$  KO producing elimination of functional  $\alpha 9^*$ -nAChR, either leading to immune cell quiescence, could account for the attenuated disease response and suppression of immunity and inflammation.

As to be expected, our findings in the current study pose new questions that await future investigation. First, it remains to be determined whether deletion of specific nAChR subunits leads to compensatory changes in others, although RT-PCR studies do not indicate dramatic changes in levels of other nAChR subunit messages in  $\alpha 7$  KO mouse immune cells, brain, or brain microglia (Hao et al., 2011). Second, there could be influences of disease state on nAChR subtype and subunit expression, also as suggested by our earlier work (Hao et al., 2011). Third, expression of nAChR that mediate natural effects of ACh and/or summed protective effects of nicotine in different immune cell types is incompletely understood. For example, it is reported that nAChR  $\alpha 7$  and  $\alpha 9$  subunits are expressed by CD4+ and CD8+ T cells (Qian et al., 2011), consistent with our observations (Hao et al., 2011). Moreover, regulatory T cell responses appear to be dependent on  $\alpha 7$ -nAChR (Wang et al., 2010). Infiltrating macrophages or microglia in the brain are also key players in EAE pathology, and the expression profile of  $\alpha 9^*$ -nAChR and  $\alpha 9$  subunits in these cells remains to be elucidated. Last, nAChR subtypes respond differently to nicotine and at different concentrations. Although the range of non-toxic, but behaviorally-relevant doses of nicotine that can be studied in mice or other animals *in vivo* is limited, use of other agents selective for particular nAChR subtypes could help elucidate their contributions to inflammatory and immune responses.

In summary, our observations motivate strategies involving nAChR subtype-selective compounds to modulate responses involved in inflammatory diseases. Recognizing that much more work is to be done before applying our findings to human disease conditions, we think that therapeutic possibilities are realistic and proximal. For example,  $\alpha 9^*$ -nAChR of disease relevance appear to be situated in peripheral immune cells, allowing for design of ligands targeting them that would not be required to penetrate to the brain, elevating the likelihood that adverse side effects could be well-controlled. Moreover, there is limited expression of  $\alpha 9^*$ -nAChR in other tissues (in olfactory epithelia and the cochlea and in some skin cells; Jensen et al., 2005; Lukas and Bencherif, 2006; Taly et al., 2009) relative to their



rich expression in the immune system. Also, auditory function in  $\alpha 9$  KO animals (but not in  $\alpha 9/\alpha 10$  DKO mice) is like that in WT mice (Morley et al., 2017a), suggesting a low risk of non-immune system side effects if  $\alpha 9^*$ -nAChR are modulated. Nicotine itself is a therapeutic candidate, as it is likely to be much safer delivered as a medicine than via use of tobacco products and has very limited abuse liability on its own in humans (West et al., 2000). There do seem to be links between elevated MS susceptibility and progression and levels of tobacco cigarette smoking (Handel et al., 2011), especially if smoking occurs in adolescence (Salzer et al., 2013). However, other studies indicate that nicotine actually could be protective in MS (Nizri et al., 2009; Shi et al., 2009); as opposed to effects seen in cigarette smokers, MS susceptibility seems to be reduced in users of snuff (Hedström et al., 2009, 2013). Nicotine exposure does not equate to cigarette smoking, which brings exposure to thousands of other harmful compounds. Thus, all things considered,  $\alpha 9^*$ -nAChR are viable targets for development of superior approaches to block or control inflammation and immunity. On the other hand,  $\alpha 7^*$ -nAChR activation could protect against inflammatory and autoimmune insults if activated by  $\alpha 7^*$ -nAChR-selective ligands. In the long term, findings from this and other studies

could alter clinical practice in the treatment of inflammatory and autoimmune diseases, such as MS.

## AUTHOR CONTRIBUTIONS

BJM, F-DS and RJL conceived of the study and, with QL, outlined general experiments. QL executed specific methods, acquired data and engaged in initial data analysis. QL and RJL drafted the initial version of the manuscript and refined data analysis. QL, PW, BJM, F-DS and RJL contributed to data interpretation and revised the manuscript, which was progressively edited by RJL. QL, PW, BJM, F-DS, and RJL reviewed and approved the final version of the report.

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**Conflict of Interest Statement:** F-DS and RJL are co-holders of U.S. Patent Number 8,841,329 “Nicotinic Attenuation of CNS Inflammation and Autoimmunity.”

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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