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INVITED REVIEW

Modulation of inflammatory pathways by the immune cholinergic system

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Abstract Research done in the past years pointed to a novel function of cholinergic transmission. It has been shown that cholinergic transmission can modulate various aspects of the immune function, whether innate or adaptive. Cholinergic transmission affects immune cell proliferation, cytokine production, T helper differentiation and antigen presentation. Theses effects are mediated by cholinergic muscarinic and nicotinic receptors and other cholinergic components present in immune cells, such as acetylcholinesterase (AChE) and cholineacetyltransferase. The α7 nicotinic acetylcholine receptor was designated anti-inflammatory activity and has shown promise in preclinical models of inflammatory disorders. We herein describe the various components of the immune cholinergic system, and specifically the immune suppressive effects of α7 activation. This activation can be accomplished either by direct stimulation or indirectly, by inhibition of AChE. Thus, the presence of the immune cholinergic system can pave the way for novel immunomodulatory agents, or to the broadening of use of known cholinergic agents.

Keywords Multiple sclerosis · Myasthenia gravis · Neuroinflammation · Acetylcholinesterase inhibitors · α7 nicotinic acetylcholine receptor

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Introduction

Acetylcholine (ACh) is mainly perceived as a neurotransmitter. It can be found in brain areas associated with cognitive function such as the hippocampus, amygdala and cerebral cortex. It has a central role in the autonomic system: it is the main neurotransmitter in the preganglionic neurons in both the sympathetic and parasympathetic systems and the post-ganglionic neurotransmitter in the parasympathetic system. In neurons, ACh is also responsible for motor function, as it is present in the neuromuscular junction (NMJ). ACh is synthesized by the enzyme cholineacetyltransferase (ChAT), and packed into vesicles in the neuronal terminus. It is released to the synaptic space upon neuronal activation, and then interacts with its receptors. Cholinergic receptors can be divided to muscarinic and nicotinic (mAChRs and nAChRs, respectively). mAChRs are G protein-coupled receptors. They comprise of five subtypes. M_1, M_3 and M_5 are coupled to G_q receptors, whereas M_2 and M_4 are coupled to G_i receptors (Langmead et al. 2008). nAChRs are pentameric receptors comprised of different α, β, γ, δ, and ε subunits. There are nine α subunits and four β subunits, allowing for various combinations with different physiological function and ligand affinity (de Jonge and Ulloa 2007). The termination of signaling in the cholinergic system is achieved by degradation of ACh by acetylcholinesterase (AChE). This enzyme has several isoforms, all of which are a product of alternative splicing of a single gene. Exon 2 encodes for the catalytic activity of the enzyme and is common to all isoforms (Grisaru et al. 1999). However, recent findings demonstrate that the cholinergic system is not restricted to neurons and synapses, but may also involve other system such as the urinary system, keratinocytes, bronchial system and more

(Kawashima and Fujii 2003). Here we will focus on the effects of immune cholinergic system.

The non-neuronal immune cholinergic system

It has been shown that resting lymphocytes and macrophages posses a complete cholinergic system, which includes the ability to synthesize and degrade ACh (by ChAT and AChE, respectively) (Fujii et al. 2003). Activation of immune cells increased their ability to synthesize and secrete ACh by increased ChAT expression, as shown by measurement of extracellular levels of ACh (Fujii et al. 1996). T cells also express various subtypes of muscarinic and nicotinic cholinergic receptors (Sato et al. 1999): all subtypes of mAChR were shown to be expressed on blood bank donors, although not consistently. nAChR muscle type subunits, i.e., $\alpha 1$, $\beta 1$ and ε, were not expressed by the same cells. We could not detect them even after immune activation either by antigen or by a mitogen (Nizri et al. 2006). Among the neuronal type, $\alpha 2$, $\alpha 5$ and $\alpha 7$ were the most consistently expressed. These data are summarized in Table 1. It was also shown that activation of T cells, either $CD4^+$ or $CD8^+$ cells altered the expression of cholinergic receptors. T-cell receptor (TCR) activation of $CD4^+$ T cells induced the expression of $\alpha 4$ nAChR and upregulated the expression of $\alpha 5$, $\alpha 10$, $\beta 4$, M1 and M5 cholinergic receptors. Moreover, differentiation of T cells toward a specific T helper (Th) lineage (see below) was also accompanied by alteration of the cholinergic receptor repertoire (Qian et al. 2011). Thus, T-cell cholinergic receptor repertoire is a dynamic one, influenced by T-cells' activation state and Th commitment. Murine macrophages and microglia were also shown to express $\alpha 7$ RNA and protein (Shytle et al. 2004; Wang et al. 2003). Mouse B cells were shown to express $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$ and $\beta 4$ nAChR subunits. The number of $\alpha 7$ subunits increased with B-cell maturation, while $\alpha 4$ and $\alpha 5$ were expressed in immature B cells (Skok et al. 2007).

Cholinergic signaling and immune cells

The effects of the cholinergic system on immune cells can be divided into the effects on cells participating in the innate immune response and to those participating in the adaptive immune response. This distinction has implication for the possible therapeutic potential of cholinergic agents, as they could be used in immune perturbations of both classes.

Cholinergic effects on cells of the innate immune response

Macrophages

ACh was shown to decrease pro-inflammatory cytokine production by activated macrophages. Murine macrophages were activated by lipopolysaccharide (LPS), an endotoxin produced by all gram-negative bacteria and implicated in the pathogenesis of septic shock, to secrete TNF-α, IL-1β and IL-6, all pro-inflammatory cytokines. ACh at the micromolar range significantly reduced this cytokine secretion. ACh had no effect on the secretion of IL-10 (Borovikova et al. 2000). The effects of ACh could be recapitulated by nicotine, but not by muscarine. Subsequent work done by the same group (Wang et al. 2003) indicated that the $\alpha 7$ nAChR was involved in this anti-inflammatory effect of nicotine, since it was abolished in $\alpha 7^{-/-}$-derived macrophages or wild-type (WT) macrophages treated with anti-sense to the $\alpha 7$ mRNA. The mechanism implicated in this effect was reduced NF-κB-mediated transcription. Application of anti-sense to the $\alpha 5$ subunit, could not suppress the nicotinic effects of macrophages. In another report by the same group, it was shown that nicotinic $\alpha 7$ agonist suppresses pro-inflammatory cytokine production in response to various toll-like receptor (TLR) ligands such as TLR2, TLR3, and TLR9 agonists (Rosas-Ballina et al. 2009). The effect on NF-κB-mediated transcription points to a powerful anti-inflammatory activity of this cholinergic

Table 1 Expression of muscarinic receptor subtypes and nicotinic receptor subunits mRNA by human T and B cell lines

Cell line	Muscarinic subtype					Nicotinic receptor subunits								
	m1	m2	m3	m4	m5	$\alpha 2$	$\alpha 3$	$\alpha 4$	$\alpha 5$	$\alpha 6$	$\alpha 7$	$\beta 2$	$\beta 3$	$\beta 4$
CEM (T)	+	−	+	+	+	−	+	−	+	+	+	−	−	+
HPB-ALL (T)	−	+	+	+	+	+	−	−	+	+	+	−	−	+
HUT-78 (T)	+	+	+	+	+	+	−	−	+	+	−	−	−	−
Jorkat (T)	−	−	−	+	+	−	−	−	−	−	−	−	−	+
MOLT-3 (T)	−	−	+	+	+	−	+	−	+	+	−	−	−	+
BALL-1 (B)	+	−	−	+	+	−	−	−	+	−	−	+	−	+
Daudi (B)	−	+	+	+	+	+	−	−	+	+	+	−	−	−
NALM-6 (B)	−	−	−	+	+	−	−	−	+	−	−	+	−	+

pathway, as many other pro-inflammatory mediators are transcribed through it (Barnes and Karin 1997). Indeed, studies by the same group revealed decreased high-mobility group box 1 protein (HMGB-1) secretion after LPS activation of macrophages. This protein was later implicated as a late effector in the septic cascade (Lotze and Tracey 2005). The same effects of nicotine could be demonstrated on microglia, the brain-residents macrophages (Shytle et al. 2004). Microglia express both α7 nAChR mRNA and protein. Its activation with nicotine or ACh, again in the micromolar range, on LPS-stimulated microglia, resulted in about 50% TNF-α reduction. This was mediated through MAP-kinase inhibition.

Dendritic cells

Until now, there is no direct evidence for nAChR expression on dendritic cells (DCs). We and others have shown decreased antigen presentation following cholinergic activation (Nizri et al. 2008, 2009). It was also shown that co-stimulatory molecules such as CD80, CD86 and MHCII expression on antigen presenting cells (APCs) is reduced upon nicotine treatment (Nouri-Shirazi and Guinet 2003). However, these results were obtained following in vivo treatment with nicotine, which could attest to an indirect effect of nicotine on DCs either through other cell types such as T cells, or through corticosteroids, whose secretion is known to be elevated after nicotinic stimulation (Seyler et al. 1984). However, in vitro treatment of human dendritic cells with nicotine decreased their phagocytic activity and pro-inflammatory cytokine secretion. DCs treated with nicotine failed to mount an effective Th_1 response in T cells. This points to a direct effect of nicotine on DCs, but still does not point to a specific molecular target (Nouri-Shirazi and Guinet 2003; Nouri-Shirazi et al. 2007). In light of the aforementioned effects of α7 nAChR on immune cells, it seems plausible that these effects are mediated also by the same receptor.

Cholinergic effects on cells of the adaptive immune response

T cells

T-cell proliferation is a key step according to the clonal immune theory, in which the T cell expressing the receptor that identifies the pathogen or immune activator, proliferate to create the active immunological clone.

Nicotine and ACh in the micromolar range could inhibit T-cell proliferation in response to mitogen. The same effect could be reproduced by carbamyl chloride (CC), a cholinergic non-specific agonist (Nizri et al. 2006). The concentration used in these experiments is similar to that described for macrophages' cholinergic suppression (Borovikova et al. 2000). Interestingly, muscarinic activation could increase T-cell proliferation, and when muscarinic blockers such as atropine were used in conjunction with cholinergic agents, they increased the inhibitory effect of the later (Nizri et al. 2006). Accordingly, it was reported that mAChR activation increased the production of pro-inflammatory mediators by enhancement of c-fos and iNOS transcription (Fujii and Kawashima 2000). Thus, cholinergic stimulation could mediate pro or anti-inflammatory effects based on muscarinic or nicotinic receptor activation, respectively. Interestingly, when cholinergic agents such as AChEIs were applied to stimulated T cells, proliferation was inhibited in an α7 nAChR-dependent mechanism. Rivastigmine or EN101 (an anti-sense to exon 2 of AChE) suppressed T-cell proliferation, and this suppression was abolished in the presence of α7 nAChR blockers, whether pharmacological or anti-sense (Nizri et al. 2006, 2008) (Fig. 1). We have shown that inhibition of AChE caused extracellular increase in ACh concentration that subsequently induced cholinergic receptor activation. This mechanism is similar to the therapeutic mechanism by which AChEIs function in myasthenia gravis (MG) or Alzheimer's disease (AD).

The α7 nAChR is expressed by naïve T cells, as was described for macrophages. Its expression is augmented following immune activation (antigen or mitogen induced)

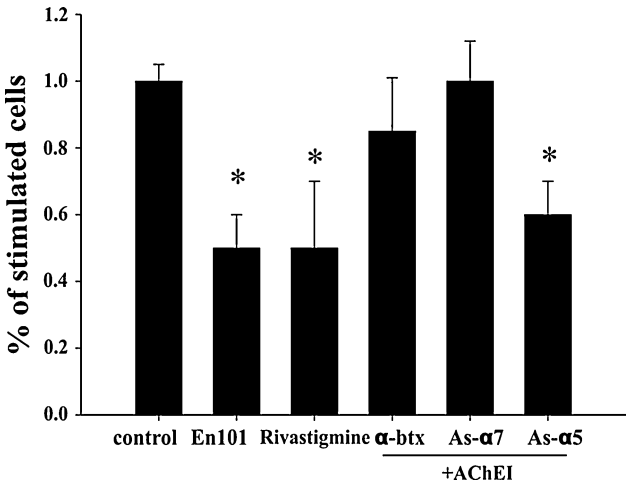

Fig. 1 AChEIs suppress T-cell proliferation in an α7nAChR-dependent mechanism. EN101, an anti-sense inhibitor of AChE and rivastigmine, a marketed anti-AD drug, inhibited T-cell proliferation to mitogens. This inhibition was abolished in the presence of α-bungarotoxin, an α7nAChR blocker and in the presence of anti-sense to α7nAChR, but not α5nAChR. *$p < 0.05$. See Nizri et al. (2006) for experimental details. In brief: Peripheral blood human lymphocytes (PBLs) isolated from blood bank donors were incubated in 96-well microtiter plates (2×10^5 PBLs in 0.2 ml RPMI and 5% FCS), in the presence of the various AChEIs and incubated for 72 h in the presence of 10 μg phytohemagglutinin

in both mRNA and protein level (Nizri et al. 2006, 2007a, 2009). Cloning of this receptor demonstrated 99.7% identity to the neuronal α7 (Razani-Boroujerdi et al. 2007). This apparent identity should not deceive: it was demonstrated that for Ca^{2+} channels even a single amino acid substitution may change permeability and function of the channel (Matza and Flavell 2009). The specific electrophysiological activity of the α7 nAChR in T cells was not investigated. This receptor-coupled Ca^{2+} channel generates rapid Ca^{2+} influxes; however, it is possible that immunological function depends on release of intracellular Ca^{2+} stores, rather than Ca^{2+} extracellular influx (Tracey 2009). Further research is needed to elucidate the intracellular effects following α7 nAChR activation.

One key aspect of $CD4^+$ T-cell function is their lineage identity. In 1986, Mosmann et al. (1986) initially proposed a model whereby $CD4^+$ T cells are subdivided into two independent subsets with distinct effector functions. Th_1 and Th_2 subsets are divided on the basis of cytokine expression and bioactivities as well as helper function. Th_1 cells secrete predominantly IFN-γ, IL-2, IL-3 and TNF-α, and control cell-mediated functions such as the activation of macrophages, while Th_2 cells secrete IL-4, IL-5, and IL-13 and lead to the stimulation of humoral immunity. Recently, a novel Th subset was defined, the Th_{17}. This newly defined Th subset was implicated in various autoimmune diseases, such as inflammatory bowel disease, collagen-induced arthritis and experimental autoimmune encephalomyelitis (EAE, see below) (Langrish et al. 2005). IL-17 is a pro-inflammatory cytokine which drives the secretion of other pro-inflammatory cytokines (such as IL-1, IL-6 and G-CSF) and chemokines from endothelial cells, stromal cells and fibroblasts (Gutcher and Becher 2007). IL-17 is secreted by a specific CD4 subset, along with IL-22, TNF-α, IL-6 and IL-23 (Liang et al. 2006). Th lineage differentiation is associated with specific transcription factors (TFs). These are crucial to the development of Th lineage, as determined by genetic targeting models. T-bet is the TF responsible for Th_1 differentiation and acts by remodeling of chromatin at the IFN-γ promoter. GATA-3 has the same function in Th_2 lineage, affecting the IL-4 promoter (Murphy and Reiner 2002). For Th_{17} subset it is the orphan nuclear receptors ROR-γt and ROR-α (Yang et al. 2008).

Activation of the α7 nAChR by nicotine decreases Th_1 (such as TNF-α, IFN-γ, IL-2) (Nizri et al. 2008, 2009) and Th_{17} (IL-17, IL-21 and IL-22) (Nizri et al. 2009) cytokine production. Nicotine decreased T-bet, but not ROR-γT or ROR-α, transcription. This may imply modulation of Th_1 differentiation while the Th_{17} lineage is affected at the activity but not on the differentiation level. Nicotine increased the production of IL-4, a prototype Th_2 cytokine, and accordingly, GATA-3 transcription. Overall, it seems from this work that α7 nAChR suppresses the Th_1 and Th_{17} lineages while augmenting Th_2 lineage activity. However, a recent work in experimental inflammatory bowel disease described nicotine suppression of the Th_2 lineage activity, and an increase in Th_1 and Th_{17} activity (Galitovskiy et al. 2011). Importantly, this effect of nicotine was correlated to the expression of the α7 nAChR: the inhibitory effects of nicotine on Th_2 lineage were correlated with high level of α7 expression on these T cells, whereas the failure to suppress Th_1 lineage was accompanied by low expression of α7 nAChR. This implies that nicotine does not imminently suppress a specific Th lineage, but rather may inhibit any active lineage as long as there is α7 expression on these cells.

$CD8^+$ T cells are implicated in various biological processes, among them immunity against viral pathogens and graft rejection. A commonly used model for T-cell alloreactivity is the mixed lymphocyte reaction model. In this model, recognition of alloantigen T cells generates IL-18 production which further augments various co-stimulatory and adhesion molecules expression along with IFN production. It was shown that nicotine inhibits this over expression of co-stimulatory and adhesion molecules, and also suppresses inflammatory cytokine production. These effects on T cells were abolished in the presence of specific α7 nAChR antagonists and hence were attributed to the α7 nAChR (Takahashi et al. 2007).

Over all, α7 nAChR stimulation was shown to modulate T-cell activity extensively including T-cell proliferation, cytokine production and specific T-cell proliferation and function. The effects of cholinergic stimulation were demonstrated on both $CD4^+$ and $CD8^+$ T cells. Another important T-cell subset affected by nicotine is regulatory T cell ($FOXP3^+CD4^+CD25^+$). Preliminary data show that treatment with nicotine increase their number (Nizri et al. 2009), however, delineation of specific function of these cells under cholinergic modulation is lacking.

B cells

Nicotine was shown to affect the size of the B-cell niche in bone-marrow (BM) and this effect was β2 dependent, as the number of B cells in BM was affected in $\beta 2^{-/-}$. However, this receptor did not affect the number of B cells in the spleen, where the α7 nAChR was the dominant receptor. This data implicates different expression of cholinergic receptors in different anatomic sites. This may point to different effects of cholinergic modulation depending on anatomic site.

Mice deficient in one of the nAChR subunits had less serum IgG and less IgG producing cells. However, cells from deficient animals had larger antibody production in response to activation, due to increased CD40 expression.

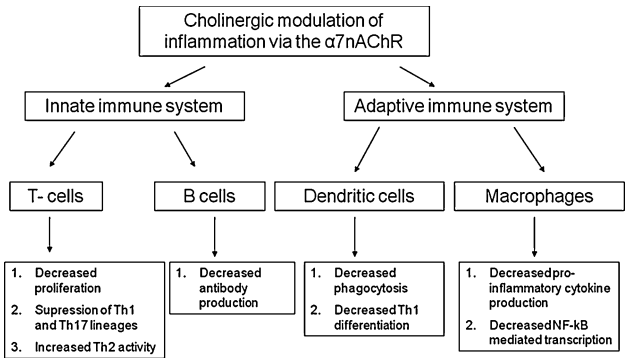

Fig. 2 Cholinergic modulation of inflammation. The various pathways by which cholinergic transmission affects inflammation are listed. See text for details and references

Interestingly, the inhibitory effects of nicotine on antibody production could only be demonstrated in the absence of the $\beta 2$ subunit (Skok et al. 2007). In another study (Fujii et al. 2007), $\alpha 7$-deficient mice had increased serum IgG1 levels and increases specific IgG1 production in response to antigen. This increase was accompanied by enhanced production of IL-6, TNF-α and IFN-γ of T cells from the same mice. The increased antibody production in this system may be attributed to the effect of $\alpha 7$ on CD4 T helper cells which induce B-cell antibody production. Overall the $\alpha 7$ activation also inhibits antibody production.

Thus cholinergic modulation of inflammation spans both the adaptive and innate immunes systems, across various cells and mechanisms (Fig. 2).

Cholinergic signaling in immune responses: paracrine or nerve-driven regulation?

The existence of the immune cholinergic system paves the way for nerve–immune interactions. Indeed initial work demonstrated that vagus nerve activation was the source for ACh which later on stimulated the $\alpha 7$ nAChR. The liver was first implicated in the location of the neural-immune "synapse". It was shown that liver TNF-α production differed between control and vagotomized animals. This production was suppressed following vagal stimulation (Borovikova et al. 2000). Subsequent work by the same group identified the spleen as the major site of nerve–immune interaction. Specific (dorsal branch) vagal transection abolished the effects of vagal nerve stimulation on pro-inflammatory cytokine production (Huston et al. 2006). This vagal branch innervates the celiac plexus and indirectly, the spleen. These results were further confirmed in splenectomized animals: vagal innervation of the spleen mediated its anti-inflammatory activity (Huston et al. 2006; Rosas-Ballina et al. 2008).

These and other findings led to the definition of the "cholinergic anti-inflammatory reflex", in which the nervous system takes a part in the control of the magnitude and quality of immune response. The afferent arm of the reflex consists of vagal afferents which are activated due to inflammation initiated by pathogen-associated molecular patterns (activators of TLRs), pro-inflammatory cytokines (such as IL-1β) or endogenous markers of damage like HMGB-1 (Tracey 2009). Such a pathway was demonstrated in the initiation of sickness behavior in rats. When IL-1β is injected intraperitonealy, it initiates sickness behavior in animals, manifested as fever, anorexia, acute phase reactions and decreased arousal. This effect is abolished in vagotomized rats, pointing to the vagus nerve as the afferent route for initiation of sickness behavior. It seems that the glomus cells present in the vicinity of vagal afferents sense IL-1 and release dopamine which stimulates vagal action potentials (Niijima 1996). This neural network provides the CNS with the ability to monitor inflammatory reactions occurring in epithelial compartments, and also to initiate a neural reaction to this process. The efferent arm of this reflex is efferent vagal neurons which release ACh on sites of the reticulo-endothelial system and modulate both macrophages and T-cell activity. Until recently the specific source of ACh in the spleen was undetermined: although ACh levels can be easily measured in the spleen, cholinergic nerve endings were never identified, despite intensive efforts. On the other hand, catecholaminergic innervation of the spleen is well described, including close contact between T cell and macrophages and catecholaminergic neuron endings (Rosas-Ballina et al. 2008; Tracey 2009). It was recently shown that cholinergic production in the spleen depends on a specific population of $CD4^+$ memory T cells. These cells reside in close proximity to adrenergic nerve endings innervated by vagal nerve terminals and express both adrenergic receptors and the ability to produce ACh, namely ChAT. Thus, the efferent reflex arm actually entails vagal activation of specific T cells by post-ganglionic adrenergic fibers. Indeed, in nude mice, vagal stimulation does not inhibit TNF-α production, while reconstitution of this population to nude mice reactivates the neuro-inflammatory reflex (Rosas-Ballina et al. 2011). Interestingly, these T cells were $CD44^{high}$–$CD62^{low}$ and from the Th1 and Th17 lineages, and not regulatory T cells, according to immunophenotyping. These are the pro-inflammatory lineages who become suppressors of inflammation upon vagal innervation. Further characterization of these induced regulatory cells is expected.

However, as previously described, immune cells posses the ability to synthesize ACh by themselves and that this production is augmented following immune activation. This may point for a role of ACh as internal mediator of immune function, similar to cytokines and other immune

active molecules. Of note, ACh existence in evolution preceded the appearance of the CNS. There is a possibility that it arose first as an inflammatory mediator in immune response (Fujii et al. 2003).

The existence of the immune cholinergic system highlights novel targets for pharmacological intervention. Research done by our group utilized AChEIs and nicotinic agonists for the modulation of neuroinflammatory conditions with success in pre-clinical models (Nizri et al. 2005, 2006, 2007a, 2008, 2009). Others have shown the efficacy of nicotinic agonists or cholinergic stimulation of immune cells in various diseases including septic endotoxemia model (Huston et al. 2007), collagen-induced arthritis (van Maanen et al. 2009), pancreatitis (van Westerloo et al. 2006) and colitis (Ghia et al. 2007). Further research is needed to apply these opportunities clinically.

Involvement of cholinergic transmission in clinical and experimental disease states

As outlined above, cholinergic transmission stimulators and agonists were recently exploited in various inflammatory conditions, induced by the innate or the adaptive immune system [reviewed in (Tracey 2009)]. Here we will focus on the most extensively studied models.

Experimental endotoxemia and septic shock

Sepsis is the most common cause of death in intensive care units, and despite decades of clinical and pre-clinical research, little progress has been made in the treatment of this disease. Sepsis is defined as systemic inflammatory reaction syndrome (SIRS) in the presence of suspected or proven infection (Russell 2006). It is a systemic response manifested as fever, tachycardia, leukocytosis or tachypnea which stems from the interaction between pathogens, usually gram negative bacteria, and the innate immune system. Activation of this system by pathogen-associated molecular patterns and their cognate receptors on innate immune cells induces secretion of pro inflammatory cytokines and mediators. These further induce activation of neutrophils, macrophages and platelets, overall culminating in a cytokine storm and immune over-activation (Russell 2006).

The experimental endotoxemia model is a commonly used model of sepsis. In this model experimental animals are injected either intravenously or intraperitonealy with endotoxin (LPS), inducing a shock state accompanied with immunological activation. It was demonstrated that vagus nerve stimulation increased animals' survival and mean arterial pressure in this model. Vagotomized animals were more susceptible to death than sham-operated animals (Borovikova et al. 2000). Subsequent work by the same group utilized vagus nerve stimulator, a device in clinical use for the treatment of intractable epilepsy, to treat sepsis in experimental animals. Again, vagus nerve stimulation with transcutaneous device reduced both serum pro-inflammatory levels and animals' mortality (Huston et al. 2007).

The use of the vagus nerve may not be limited only for anti-inflammatory interventions but also for the assessment of the inflammatory set point in individuals. It was shown that heart rate variability is a measure of vagal activity. A correlation could be demonstrated between heart rate variability and the tendency to develop inflammatory diseases and their severity. For example, it was shown that vagal activity as measured from heart rate variability in septic patients admitted to intensive care unit was correlated with survival, length of hospital stay and complications (Pontet et al. 2003). Similar findings were observed for several auto-immune and inflammatory conditions (Tracey 2009).

Alzheimer's disease

Alzheimer's disease is the most common dementia comprising about two-thirds of all diagnosed dementias. The cognitive deterioration is manifested as a decline in memory, judgment, language, decision-making and orientation to surroundings (Nussbaum and Ellis 2003). Pathologically, the disease is characterized by neuronal and synaptic loss in the cortex and hippocampus, both areas associated with cognitive function. Another area which typically degenerates early in the disease is the basal nucleus of Meinert, which is a major source of cortical cholinergic input. Therefore, AD is almost invariably associated with a disruption of cholinergic balance. Extracellular plaques containing β-amyloid and intracellular neurofibrillary tangles containing hyperphosphorylated tau protein accompany, and may actually induce, neuronal loss (Desai and Grossberg 2005). There is also prominent activation of astrocytes and microglia near the plaques, attesting to an innate immune response (Monsonego and Weiner 2003). Recently, a T-cell dependent component in AD was identified (Monsonego et al. 2003).

To date, there is no treatment that affects AD pathogenesis, and hence treatment is mainly symptomatic. In early stages, centrally acting AChEIs are used in an attempt to restore cholinergic input and partially ameliorate the memory loss (Cummings 2004). These drugs penetrate the blood–brain barrier and act by enhancing cholinergic transmission in brain areas associated with cognitive

functions and suffering from neuronal loss. Clinical observations suggest that there may be additional mechanisms by which AChEIs act in AD (Giacobini 2003). We suggested that AChEIs may act as anti-inflammatory agents (Nizri et al. 2006, 2007b).

As noted above, there is an inflammatory component in AD. Inflammation in AD seems to be a double-edged sword. On one hand, activation of the adaptive immune system against β-amyloid, either by active or passive immunization, constitutes a strategy for AD therapy (Bard et al. 2000; Schenk et al. 1999). Further along this line, it was also found that mice deficient in complement activity were affected to a greater extent by amyloid deposits, implicating a beneficial activity of the innate immune system (Wyss-Coray and Mucke 2002). On the other hand, it seems that although the primary activation of the immune system is intended to clear the amyloid plaques, when clearance fails, the chronic over-activation of the inflammatory process becomes detrimental (Akiyama et al. 2000; Wyss-Coray and Mucke 2002). Indeed, various epidemiological studies revealed an inverse relationship between the use of anti-inflammatory agents and AD (Akiyama et al. 2000). Despite these findings, clinical trials with non-steroidal anti-inflammatory drugs [both cyclooxygenase (COX)-1 and COX-2 inhibitors] (Aisen et al. 2003; Scharf et al. 1999) or with prednisone (Aisen et al. 2000), reported negative outcomes. A randomized-controlled trial of primary prevention of AD using celecoxib (selective COX-2 inhibitor) or naproxen (non-selective COX inhibitor) in the treatment arms, reported negative results (Martin et al. 2008). There was even a tendency of treatment with naproxen to become detrimental. Thus, anti-inflammatory treatment of AD as a sole modality is questionable.

Nevertheless, in lieu of the inhibitory effect of ACh on pro-inflammatory cytokine production by microglia described above (Shytle et al. 2004), the loss of cholinergic transmission described in both aging and in AD may favor an activated state of microglia.

Astrocytes also express α7 nAChR, and this expression is up-regulated in the brains of AD patients (Teaktong et al. 2003). Therefore, it is possible that AChEIs affect astrocytes and microglia in the same way as they affect T cells: increasing the interaction of ACh with α7 nAChR, and so harness the anti-inflammatory effects of this receptor. Indeed, evidence from human subjects using AChEIs points to an immunomodulating effect of these drugs. Long-term AChEIs treatment induced a Th1 to Th2 shift expressed by prototype cytokine production (Reale et al. 2004, 2006). Thus, AChEIs may affect the inflammatory activity of various cell types participating in AD-associated inflammation. This, in turn, could affect neuronal loss and cognitive function.

Experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis is a widely used model for the study of multiple sclerosis (MS). MS is an inflammatory disease of the CNS. It is the most common cause for neurological disability in the young adults (Sospedra and Martin 2005). The clinical manifestations usually include fatigue, muscle weakness, spasticity, gait and bladder dysfunction, vision abnormalities, cognitive and affective disorders (Kesselring and Beer 2005). The cognitive dysfunction in MS consists of memory and attention impairment, reduced speed of information processing and a decrease in verbal fluency (Gilchrist and Creed 1994). EAE is an inflammatory disease of the CNS in which myelin components are the focus of the autoimmune attack. Since the disease is induced by a known antigen, study of the pathogenesis of EAE has led to many immunological insights, allowing lessons from EAE to be generalized and applied to other autoimmune diseases (Steinman and Zamvil 2006). $CD4^+$ T cells are sensitized in the periphery against protein components of the myelin sheath, such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP). These encephalitogenic T cells then migrate to the CNS and, upon additional activation by resident antigen-presenting-cells (APC) such as microglia, astrocytes and a subpopulation of dendritic cells (Greter et al. 2005), initiate plaque formation. Damage mechanisms in the CNS include secretion of cytokines by pathogenic T cells, mainly of the Th_1 and Th_{17} lineages (see below), but also activation of glial cells to secrete both pro-inflammatory cytokines and inflammatory mediators such as nitric oxide (NO). Overall, this process leads to destruction of the myelin sheath, axonal damage and even loss (Hemmer et al. 2002). The clinical manifestation of this process is neurological motor dysfunction that can be quantified using a specific score. The $MOG_{35–55}$-induced model of EAE is characterized by an acute inflammatory phase, followed by a chronic phase of neurological deficit. This pattern is more compatible with the common course of MS.

The clinical neurologic deficit is accompanied by specific alterations in histological sections from spinal cord and brain tissue. These include inflammatory infiltrates containing macrophages and T cells in neural tissue, together with axonal damage and loss. Moreover, based on the encephalitogenic T-cell migration pattern and the well-known disease kinetics, the model affords analysis of various immunological parameters at different phases of the disease (Hjelmstrom et al. 1998; Wolf et al. 1996).

Until recently, EAE was considered a Th_1-mediated disease. Several lines of evidence supported this notion: Th_1 cytokines (like TNF-α and IFN-γ) were up-regulated in inflammatory plaque (Traugott and Lebon 1988), Th_1-

polarized encephalitogenic T cells could adoptively transfer EAE (Ben-Nun et al. 1981) and more recently, it was shown that mice deficient in T-bet are resistant to EAE (Bettelli et al. 2004). Furthermore, data regarding IL-12 also reinforced the Th_1 hypothesis. IL-12 is secreted by APCs upon antigen presentation and plays a major role in the development of Th_1 lineage (Gutcher and Becher 2007). Structurally, it is a heterodimeric cytokine comprised of two subunits p40 and p35. Animals deficient in p40 were resistant to EAE (Segal et al. 1998), and antibodies to IL-12 ameliorated disease symptoms (Leonard et al. 1995).

However, several experimental flaws appeared in this theory. IFN-γ knock-out (KO) mice developed more severe EAE than wild-type mice (Ferber et al. 1996). The same is true for IFN-γ receptor KO animals (Willenborg et al. 1996). In fact, there were reports that IFN-γ administration ameliorated clinical signs of EAE (Voorthuis et al. 1990), whereas treatment with antibodies to IFN-γ exacerbated EAE (Billiau et al. 1988). Moreover, deletion of p35, the light chain of IL-12, did not confer resistance to EAE, but actually increased disease severity (Becher et al. 2002; Gran et al. 2002). So while p40 is essential for EAE induction, p35 is not. Indeed, it was discovered that p40 can dimerize with another subunit, p19, to form IL-23, a novel cytokine which belongs to the IL-6 super-family. Thus, the resistance of $p40^{-/-}$ mice to EAE actually reflects IL-23-deficiency phenotype. This was later confirmed in $p19^{-/-}$ mice. While these mice were resistant to EAE, they had no defect in Th_1 lineage development (Cua et al. 2003). IL-23 is secreted by APC and acts on the IL-23 receptor (IL-23R) which is composed of the p40 binding protein IL-12Rβ1 subunit and the signaling IL-23R subunit. IL-23R is present on the surface of various cells of the immune system, including activated/memory T cells, NK cells, DCs, monocytes, and macrophages. IL-23R is highly expressed on murine memory $CD4^+$ T cells and is expressed at low levels on naïve T cells, permitting unique effects of IL-23 on this cell type (Gutcher and Becher 2007). The importance of IL-23 in the development of autoimmunity stems from its role in the differentiation of IL-17 secreting T cells (Th_{17}). This newly defined Th subset was implicated in various autoimmune diseases, such as inflammatory bowel disease, collagen-induced arthritis and EAE (Langrish et al. 2005).

As could be expected from in vitro studies showing decreased Th_1 and Th_{17} reactivity under cholinergic stimulation, decreased T-cell proliferation and reduced antigen presentation by APCs, activators of the α7 nAChR suppressed effectively EAE clinical severity. We used both AChEIs as α7 activators and direct α7 agonists such as nicotine.

Treatment with various cholinergic agents affected EAE clinical severity AChEIs (Fig. 3). Cholinergic up-regulation decreased EAE severity by about 40% (Nizri et al. 2006, 2008). This effect was abolished in the presence of nicotinic blockers. Moreover, sustained release preparation increased treatment efficacy and yielded 70% reduction in EAE severity. The clinical amelioration was accompanied by improvement in histopathological parameters of damage, such as axonal loss, demyelination and microglial activation (Nizri et al. 2008). As can be seen in Fig. 3 antisense (EN101) directed to AChE, rivastigmine (the inhibitor of AChE catalytic activity) as well as nicotine markedly inhibited EAE and these effects were α7 AChR dependent (Nizri et al. 2006, 2008, 2009). Mice lacking α7 AChR developed EAE with milder severity and were refractory to nicotine administration (Nizri et al. 2009).

As rivastigmine is used in clinical practice for the treatment of cognitive dysfunction in AD, we hypothesized that it could also ameliorate memory impairment associated with EAE. We measured cognitive impairment in the Morris water maze (MWM) assay after EAE induction before appearance of clinical signs. Indeed, EAE was associated with cognitive impairment, which was absent in mice injected with adjuvant without induction of CNS inflammation. This fact can be explained by inflammatory infiltration of immune cells, culminating in axonal perturbation in brain areas associated with this function, like the hippocampus, an area known to be associated with MWM

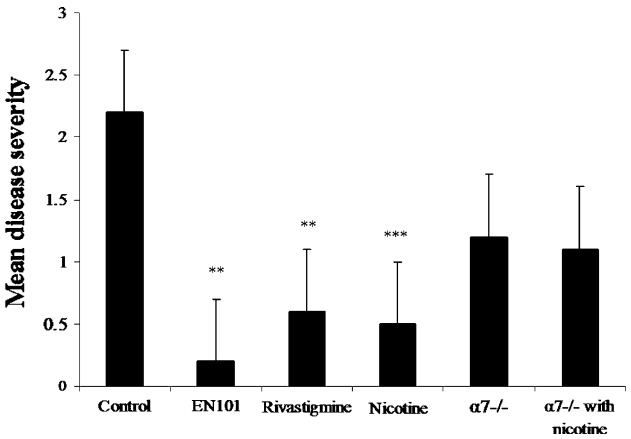

Fig. 3 Effects of various cholinergic agents on disease severity of EAE. AChEIs (such as EN101 and rivastigmine) as well as direct α7nAChR simulation by nicotine significantly decreased EAE severity. $\alpha7^{-/-}$-deficient mice had somewhat decreased EAE severity; however, nicotine did not change the disease course in these mice. $^{**}p < 0.01$, $^{***}p < 0.001$. See Nizri et al. (2008, 2009) for experimental details. EAE was induced in female C57BL/6 mice by injecting 250 µg myelin oligodendrocyte gkycoprotein (MOG) peptide 35–55 in CFA. The clinical signs of EAE were scored according to a common scale. Rivastigmine 0.75 mg/kg was administered s.c., nicotine 2 mg/kg/day was administered via ALZET implanted mini-osmotic pump

performance. Importantly, treatment with rivastigmine ameliorated performance in the MWM to naïve mice level. Analysis of hippocampal brain sections demonstrated decreased inflammatory infiltrates in rivastigmine-treated animals. The presence of cognitive impairment in mice with EAE further validates it as a model of MS, and paves the way for testing potential drugs for MS-induced cognitive dysfunction in this model. A similar beneficial effect of rivastigmine on spatial memory function was reported in acute EAE with cholinergic up-regulation and increased neuronal growth factor (NGF) production (D'Intino et al. 2005). The use of rivastigmine, an approved drug for cognitive dysfunction in MS seems plausible and recently AChEI use in MS was tested successfully in a clinical trial with donepezil (Krupp et al. 2004).

Treatment with nicotine in continuous release preparation suppressed EAE clinical score by 70%. Under this protocol, CNS infiltration by $CD4^+$ and $CD11b^+$ cells was also reduced (Nizri et al. 2009). The dose used in our experiments (2 mg/ks, s.c.) was significantly less than that used in human clinical trials in ulcerative colitis (Aisen et al. 2003). This was done to minimize side effects, which were not reported in our experiments. However, this also affords using higher doses in resistant cases. Indeed, another group reported similar effects of nicotine with 13 mg/kg in similar preparation (Nizri et al. 2009). In the same report, nicotine was also shown to inhibit the adoptive transfer form of EAE, a fact that points to the effects of the treatment on T cells.

Nicotine is known to increase corticosteroids release by activation of the hypothalamic–pituitary–adrenal axis (Seyler et al. 1984). To exclude the possibility that the effects of nicotine on EAE depend on corticosteroids, EAE in adrenalectomized mice was treated with nicotine. The effects of nicotine were not dependent on intact adrenal function, because EAE was inhibited to the same extent in the adrenalectomized mice (Nizri et al. 2009). These results are in accordance with previous published results regarding chronic nicotine treatment (Razani-Boroujerdi et al. 2007). Treatment with nicotine of $\alpha 7^{-/-}$ EAE-induced mice did not alter disease severity, indicating that the effects of nicotine depend on this receptor (Nizri et al. 2009).

Myasthenia gravis

Myasthenia gravis is a long recognized neurological disorder characterized by excessive muscle fatigability leading to moderate to profound weakness upon exertion. Basic neurophysiological and pharmacological studies pointed to malfunction of the NMJ as the underlying cause of the myasthenic weakness (Conti-Fine et al. 2006).

It is well established that the muscle fatigability in MG is caused by antibody-mediated autoimmune attack against the nicotinic AChR at the NMJ, the muscular ($\alpha 2\beta\gamma\varepsilon$) nAChR. This process abolishes the naturally occurring "safety factor" of synaptic transmission. The antibodies induce a reduction in the number of available nAChR molecules through cross linking and accelerated degradation, and possibly by complement-mediated membrane damage. These mechanisms lead to impairment of the secondary activation of voltage-gated sodium channels, which normally act as amplifiers of the end plate potential to create the muscle action potential (Conti-Fine et al. 2006). Approximately 15% of patients with MG do not have measurable antibody levels against the nAChR. A significant proportion of these "seronegative" cases have antibodies directed at the muscle specific kinase (MuSK), an NMJ protein that is associated with the AChR and plays a role in its assembly (Conti-Fine et al. 2006; Newsom-Davis 2007).

Altogether, it is fairly evident that in MG and its related disorders, "cholinergic balance" is impaired at the nicotinic synapse of the NMJ. Both the diagnosis and the symptomatic treatment of MG are based on cholinergic modulation, namely the partial restoration of cholinergic balance by the prolongation of postsynaptic receptor stimulation through AChEIs. Short-acting inhibitors of AChE such as edrophonium are used for diagnostic testing, in which the functioning of a particularly weak muscle group is shown to improve dramatically following administration of the test agent.

The current management of MG includes the use of AChEIs for temporary improvement of neuromuscular transmission, removal of anti-AChR antibodies and the use of non-specific immunosuppression or immunomodulation (Conti-Fine et al. 2006; Drachman 1994).

In experimental autoimmune myasthenia gravis (EAMG), the animal model for MG, EN-101 not only improved survival and stamina, but also improved various immunological parameters, such as antibody titer and chemokine levels in the muscles (Brenner et al. 2003). These alterations in immunological parameters are not expected if the only role of AChEIs was cholinergic up-regulation and subsequent activation of the muscular nAChR. Thus, it is possible that enhancement of cholinergic transmission in EAMG has anti-inflammatory effects, irrespective of cholinergic receptor activation.

Figure 4 summarizes the dual effects of AChEIs in human diseases and in the related experimental animal model models. In Alzheimer's disease, a local inflammatory response is present near brain amyloid plaques. Cholinergic up-regulation by AChEIs improves cognitive function and may also down-regulate inflammation by activating α7nAChR on immunocompetent cells. In the animal model for multiple sclerosis, prominent inflammatory lesions lead to demyelination and axonal loss. The

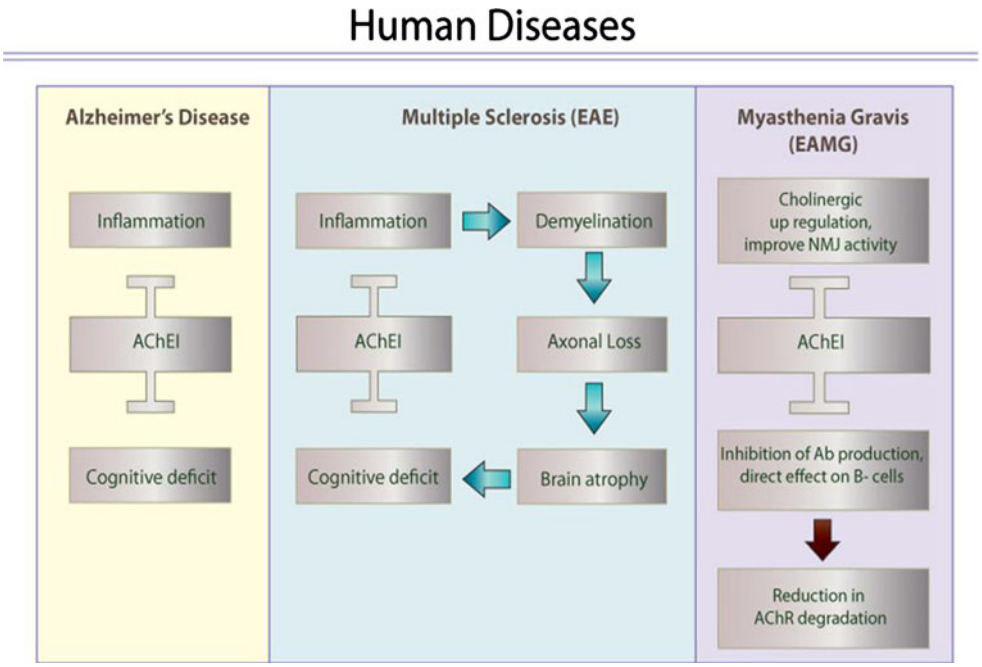

Fig. 4 In Alzheimer's disease cholinergic up-regulation by AChEIs may improve the local inflammatory response present near brain amyloid plaques. In EAE the animal model for multiple sclerosis, the cholinergic up-regulation exerted by AChEIs could be beneficial due to both inflammatory suppressing properties that may limit the axonal damage and induce an improvement of cognitive dysfunction (Nizri et al. 2006, 2008). In MG and experimental autoimmune MG (EAMG) the cholinergic balance is impaired at the NMJ. Treatments with AChEIs induce symptomatic improvement. In addition, AChEI may affect T-cell responses as well as antibody production by B cells which may lead to normalization of neuromuscular transmission and immunomodulation of the immune cells involved in disease pathogenesis (Brenner et al. 2003; Nizri et al. 2007b)

cholinergic up-regulation exerted by AChEIs could be beneficial due to both inflammatory suppressing properties that may limit the axonal damage and induce an improvement of cognitive dysfunction (Nizri et al. 2006, 2008). In MG and EAMG the cholinergic balance is impaired at the NMJ. Treatments with AChEIs induce cholinergic up-regulation and improvement of muscle weakness. In addition, AChEI may affect T-cell responses as well as antibody production by B cells which may lead to normalization of neuromuscular transmission and immunomodulation of the immune cells involved in disease pathogenesis (Brenner et al. 2003; Nizri et al. 2007b).

Summary

Cholinergic balance has appeared as a novel player in immunomodulation. Cholinergic transmission can affect every aspect of immune function including cell proliferation, cytokine secretion and lineage differentiation. It adds a neural component to the regulation of inflammation, but simultaneously, paves the way for novel pharmacological agents for immunomodulation acting at cholinergic sites.

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