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Review Article

Modulation of the extraneuronal cholinergic system on main innate response leukocytes

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ABSTRACT

The expression of elements of the cholinergic system has been demonstrated in non-neuronal cells, such as immune cells, where acetylcholine modulates innate and adaptive responses. However, the study of the non-neuronal cholinergic system has focused on lymphocyte cholinergic mechanisms, with less attention to its role of innate cells. Considering this background, the aims of this review are 1) to review information regarding the cholinergic components of innate immune system cells; 2) to discuss the effect of cholinergic stimuli on cell functions; 3) and to describe the importance of cholinergic stimuli on host immunocompetence, in order to set the base for the design of intervention strategies in the biomedical field.

1. Neuronal cholinergic system

The cholinergic system is integrated by synthesizing enzymes, such as choline acetyltransferase (ChAT, E.C. 2.3.1.6), the neurotransmitter acetylcholine (ACh); storage and transport elements, e.g., ACh vesicles (VACh) and the vesicular ACh transporter (VAChT); nicotinic and muscarinic acetylcholine receptors of ACh (nAChR and mAChR, respectively); degradative enzymes, such as acetylcholinesterase (AChE, E.C. 3.1.1.7), butyrylcholinesterase (BChE, E.C. 3.1.1.8), a non-specific cholinesterase, and the high-affinity choline transporter (CHT1) (Wessler and Kirkpatrick, 2001), which is responsible for high affinity uptake into the nerve terminal (Brandon et al., 2004). All these elements (summarized in Fig. 1) play a fundamental role in the nervous system by regulating diverse events such as proliferation, differentiation, neurogenesis, gliogenesis, neuronal maturation, plasticity, and axon development (Abreu-Villaça et al., 2011). Thus, this set of elements has been associated with basic processes of the nervous system, such as memory and learning; neuromuscular plaque activation, and the control of visceral and gastrointestinal function (Bellier and Kimura, 2011; Bentley et al., 2011; Deiana et al., 2011; Graef et al., 2011).

The ACh neurotransmitter has been conserved through evolution and it can be found in different biological systems of vastly diverse organisms (Wessler and Kirkpatrick, 2001). This molecule is widely distributed in the central and peripheral nervous systems. Furthermore,

Abbreviations: ACh, Acetylcholine; AChE, Acetylcholinesterase; ChAT, Choline acetyltransferase; CHT1, High-affinity choline transporter 1; COPD, Chronic obstructive pulmonary disease; fMLP, N-formylmethionyl-leucyl-phenylalanine; LTC_4, Leukotriene-C4; AChR, Acetylcholine receptor; mAChR, Muscarinic acetylcholine receptor; nAChR, Nicotinic acetylcholine receptor; pChAT, Peripheral variant of choline acetyltransferase; QNB, Quinuclidinyl benzilate; VACh, Acetylcholine vesicles

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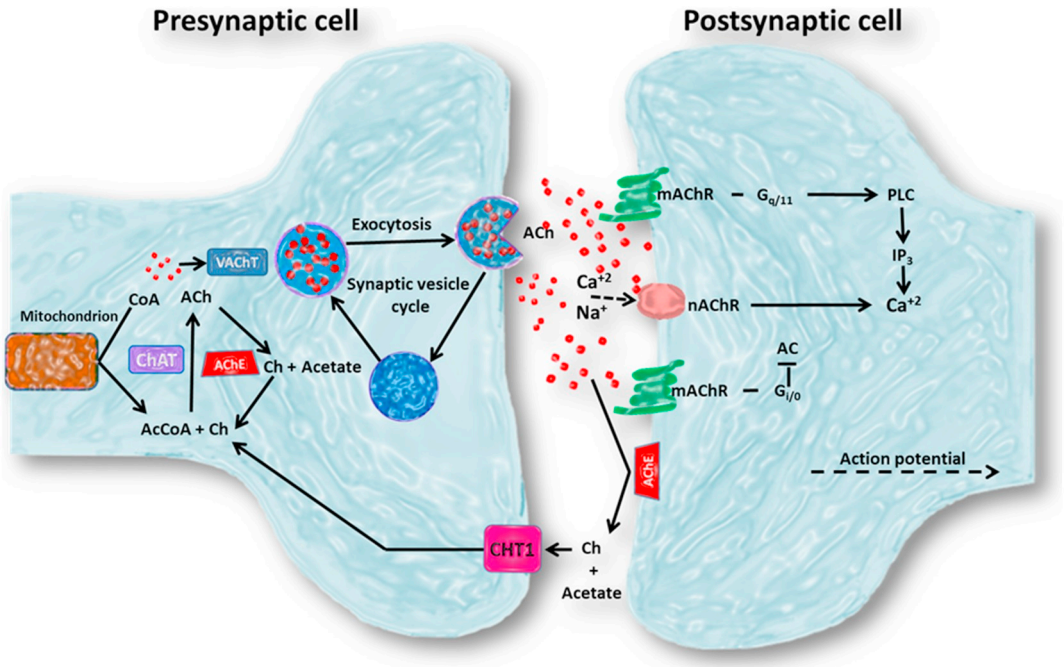

Fig. 1. Neuronal cholinergic system; ACh: Acetylcholine; AChE: Acetylcholinesterase; Ch: Choline; CHT1: High affinity choline transporter 1; ChAT: Choline acetyltransferase; CoA: Coenzyme A; AcCoA: Acetyl-Coenzyme A; VAChT: Vesicular acetylcholine transporter; mAChR: Muscarinic acetylcholine receptor; nAChR: Nicotinic acetylcholine receptor; G q/11: G q11-type protein; Gi/0: G i0-type protein; PLC: phospholipase C; IP3: Inositol 1,4.5-triphosphate; AC: Adenylate cyclase.

it is essential in the autonomous nervous system, where it is the main neurotransmitter in pre-ganglionic neurons of the sympathetic and parasympathetic systems, and a neurotransmitter in post-ganglionic neurons of the parasympathetic system (Abreu-Villaça et al., 2011; Nizri and Brenner, 2013).

Acetylcholine is synthesized in the cytosol by the ChAT enzyme by using choline and acetyl coenzyme A (acetyl-CoA) as substrates when values are 1 mM and 10 μM, respectively. The required choline for ACh synthesis comes from the diet, endosynthesis, and ACh hydrolysis, but mainly from the degradation of membrane phospholipids that contain choline (phosphatidylcholine) (Ulus et al., 1989; Taylor and Brown, 1999). Acetyl-CoA originates from pyruvate and lactate by the action of the pyruvate dehydrogenase enzyme complex in the inner mitochondrial membrane (Wessler and Kirkpatrick, 2001; Abreu-Villaça et al., 2011). There are two variants of ChAT: the common choline acetyltransferase (cChAT), which is constitutive and extensively expressed in the peripheral and central nervous system cells (Gill et al., 2007); and the peripheral variant of ChAT (pChAT) that results from alternative splicing, lacks four consecutive (exons 6–9), and is localized in the cytoplasm. This variant is preferably expressed in the peripheral nervous system and has a lower catalytic activity than cChAT. Furthermore, it has been associated with non-neuronal cholinergic functions (Bellier and Kimura, 2011).

Once synthesized, ACh is captured by VAChT, which uses a transvesicular proton gradient generated by an ATPase for the accumulation of the neurotransmitter in the VACh (Deiana et al., 2011). When ACh release is necessary, a membrane-initiated signaling cascade is activated along with soluble factors that regulate its release in a synchronized manner. This coordinated mechanism responds to the activity at postsynaptic receptors, cholinesterases and ACh levels in the extracellular milieu (Wessler and Kirkpatrick, 2001; Abreu-Villaça et al., 2011).

Upon being released into the extracellular space ACh will interact with nAChR or mAChR. These receptors are fundamental for the intracellular signaling cascade, which comprises ion flux activation, intracellular calcium mobilization, increase in cGMP levels, nitric oxide (NO) release; and activation of tyrosine kinases, small G-proteins, and mitogen-activated protein kinases (MAPK) (Wessler and Kirkpatrick, 2001; Picciotto et al., 2012). Furthermore, these cholinergic receptors play an important role in the modulation of cell proliferation and differentiation; cytoskeleton organization, gene expression regulation, and activation of ionic channels and transporters. Specifically, at the nervous system level, cholinergic receptors modulate synapse formation and maturation, axonal pathfinding and neurotransmitter release (Abreu-Villaça et al., 2011).

The nAChRs are ionotropic receptors, which form ion channels (Na^+, K^+, and Ca^{2+}). These receptors are pentameric, comprised of α (1 − 10), β (1–4), γ, δ or ε subunits, which allows a variety of functions depending on ligand affinity. In this context, there are two ways of classifying them: 1) homopentameric: these are formed by the same subunits (α7 or α9) and are extremely permeable to Ca^{2+}; 2) heteropentameric, which are formed by different subunits (α2–6 and β2–4) and are less permeable to Ca^{2+}. Once the nAChR is stimulated, Ca^{2+} can enter the cell directly through the ion channels or indirectly through the activation of voltage-dependent channels. Through the modulation of Ca^{2+} levels, nAChRs are capable of regulating cellular events such as second messenger activation and gene expression by phosphorylation of the cAMP response element-binding protein (CREB). These receptors are associated with neuronal growth, differentiation, and synapse formation during development and they are fundamental to learning and memory processes (Abreu-Villaça et al., 2011; Deiana et al., 2011).

In regard to mAChR, five genes have been identified that code five mAChRs subtypes: M1, M2, M3, M4, and M5. These receptors belong to the metabotropic receptor family and are composed of a protein with seven transmembrane domains; the third domain is intracellular and confers the characteristic function to each subtype by interacting with G-proteins. Different signaling pathways are activated depending on the mAChR subtype stimulated. M2 and M4 ($G_{i/0}$-coupled) are localized in pre- and post-synaptic neurons, inhibit adenylyl-cyclase activity (AC), which in turn reduces cyclic adenosine monophosphate (cAMP) levels; while M1, M3, and M5 ($G_{q/11}$-coupled) are localized in post-synaptic neurons, activate phospholipase C (PLC) and induce the formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 mobilizes Ca^{2+} and DAG, which in turn activate protein kinase C (PKC)

(Abreu-Villaça et al., 2011; Deiana et al., 2011; Drever et al., 2011; Carruthers et al., 2015). These receptors have been associated with effects on proliferation, differentiation, and survival of nervous system cells (Carruthers et al., 2015).

Cholinergic activity ends when ACh is hydrolyzed to choline and acetate by AChE, regulating the extracellular concentration of ACh. AChE is coded by one gene, but multiple transcripts are generated by alternative splicing. There are three isoforms of AChE: AChE-St, AChE-H and AChE-R. The tetrameric AChE-S ("S" for synapsis) is the most abundant; AChE-H is dimeric and is expressed in blood cells; and AChE-R ("R" for "red-through"), is the least frequent isoform (Abreu-Villaça et al., 2011). In addition to its enzymatic activity, AChE has important roles in the central nervous system development, such as cellular adhesion, neurite growth, circuit formation and even apoptotic processes (Abreu-Villaça et al., 2011). AChE is mainly localized in the brain, muscle, erythrocytes and cholinergic neurons (Schetinger et al., 2000), while BChE is present in serum and tissues such as the intestine, liver, kidney, heart, and lungs (Bodur and Layer, 2011; Scacchi et al., 2011). BChE can also hydrolyze ACh and even though its physiologic role is not clear, it has been reported that it can hydrolyze hydrophobic and hydrophilic compounds that contain carboxylic or phosphoric acids, respectively, acting as a possible endogenous scavenger of anticholinergic molecules (Chuiko, 2000).

Once ACh is hydrolyzed, the newly formed choline is rapidly recaptured by CHT1 and directed to the pre-synaptic cell. This supports the continuous synthesis of ACh (Brandon et al., 2004; Abreu-Villaça et al., 2011). This transporter has a high affinity for choline (Km ~1–2 μM) and its activity is influenced by a sodium electrochemical gradient. It has been demonstrated that CHT1 is primarily found in intracellular compartments, where it goes through a cycle of localization between these compartments and the cell membrane before it is internalized. The internalized CHTs are recycled and re-localized to the cell membrane by a highly regulated process that is only activated when ACh synthesis is needed (Black and Rylett, 2012).

2. Non-neuronal cholinergic system

The term "non-neuronal cholinergic system" or "extraneuronal cholinergic system" refers to the synthesis or degradation of ACh, and the response to this neurotransmitter in non-neuronal cells (Wessler et al., 1998; Grando et al., 2003; Fujii et al., 2017). That is, when epithelial, endothelial, as well as cardiovascular, digestive, urinary and immune systems cells, have the biochemical machinery to synthesize de novo and/or respond to ACh (Kawashima and Fujii, 2000; Wessler et al., 2003; Wessler and Kirkpatrick, 2008; Kakinuma et al., 2009; Beckmann and Lips, 2013). This characteristic has been identified not only in human cells but also in other vertebrates (e.g., mouse, rats and fish), lower invertebrates (e.g., sponges, corals, tunicates, sea urchins and Turbellaria), plants, fungi, protozoa, and even bacteria (Wessler and Kirkpatrick, 2008; Toledo-Ibarra et al., 2016).

In this sense, it has been reported that most non-neuronal cells have acetyl-CoA and choline, which are precursor molecules for ACh synthesis (Wessler and Kirkpatrick, 2008). Additionally, ChAT can be found in many subcellular sites, including the nucleus (Wessler et al., 2003; Abreu-Villaça et al., 2011), as seen in the central nervous system (Gill et al., 2007). In addition, the mitochondrial enzyme carnitine acetyltransferase (CarAT) is expressed in this cells, this enzyme also contributes to ACh biosynthesis (Tuček, 1982; Fujii et al., 2017). Furthermore, it has been demonstrated that non-neuronal cells also express CHT1 and have AChE activity (Wessler et al., 2003; Hanna-Mitchell et al., 2007).

The expression of nAChR and mAChR has been demonstrated in epithelial, endothelial, myocardium tissue, and immune cells, such as lymphocytes (T and B cells), macrophages and dendritic cells from humans, mice and rats. These cells express the five mAChR subtypes and they contain an array of nAChRs (Kawashima and Fujii, 2003; Kakinuma et al., 2012; Koarai et al., 2012; St-Pierre et al., 2016; Fujii et al., 2017). ACh functions are regulated through these receptors. In this way, ACh can act as a local signaling molecule, contributing to the regulation of basic cell functions (ciliary activity, water secretion, ion concentrations, mucus formation, cytoskeleton organization and cell-cell interactions) and modulation of immune functions (cytokine release, proliferation, migration, activation, and differentiation). In agreement with this, ACh can be considered a universal molecule involved in almost every cellular signaling pathway (Kawashima and Fujii, 2003; Neumann et al., 2007; Wessler and Kirkpatrick, 2008; Abreu-Villaça et al., 2011; Kummer and Krasteva-Christ, 2014).

In considering the type of immune cells, the study of non-neuronal cholinergic pathways has centered in the lymphocyte cholinergic mechanisms and less attention has been given to its role to innate immunity mechanisms. Given this background, the aims of this review are 1) to review the current information regarding the cholinergic components of the innate immune system cells; 2) to discuss the effect of different cholinergic stimuli on immune cell function; 3) and to describe the importance of cholinergic stimuli on host immunocompetence using agonists/antagonists of muscarinic and nicotinic receptors, in order to set the base for the design of intervention strategies in the biomedical field.

3. Innate immune system

The innate immune system has evolved over millennia to non-specifically control and clear invading pathogens. In order to discern between "self" and "nonself" antigens, innate cells express membrane and cytoplasmic pathogen recognition receptors (PRRs), capable of recognizing conserved antigen molecules that are widely expressed in microorganisms. These receptors are coded in the germline, that is, they do not need a genetic rearrangement prior to their expression. Thus, when an antigen enters the organism, innate immune cells immediately recognize the threat and rapidly eliminate the pathogen or contain the infection until the adaptive immune response develops. Activation of innate immune cell subsets results in the induction of an inflammatory reaction leading to the establishment of a nonspecific antimicrobial environment via the release of cytokines and the activation and recruitment of other immune cells. As such, the innate immune response is not only responsible for early pathogen containment, but also for playing a role in ensuring the adaptive immune response (Moser and Leo, 2010; Iwasaki and Medzhitov, 2015; Zhang and Liang, 2016).

Immune cell function is highly regulated by classical soluble molecules, such as cytokines, and by cell-cell interactions. Additionally, it is well established that hormones and neurotransmitters, typically produced by non-immune cells, also participate in immune system regulation (Besedovsky and del Rey, 1996; Escobedo et al., 2009; Bottasso et al., 2013). Furthermore, it is now known that immune cells not only express the necessary receptors to respond to neuro-endocrine stimuli but also have the biochemical capacity to synthesize these molecules, including ACh (Kawashima et al., 2007; Sumida et al., 2014; Fujii et al., 2017).

3.1. Innate immune cells

The arsenal of the innate immune system is composed of an array of cell subsets including phagocytes, granulocytes, and innate lymphoid-derived cells (ILCs), specialized in directly destroying the pathogen or pathogen-infected cells. In such way, innate mechanisms of immunity are essential because they are the first line of defense but also regulate the adaptive immune response. Some of the most important innate immune cells are neutrophils, monocytes/macrophages, basophils, mast cells, eosinophils, dendritic cells (DCs), and innate lymphoid-derived cells (ILCs) (Fonseca et al., 2017).

Neutrophils, which have cytoplasmic lytic granules, constitute more than 50% of the circulating leukocyte population. These cells also

produce high levels of oxygen and reactive nitrogen species (ROS and RNS, respectively). Their main antimicrobial mechanisms are phagocytosis, degranulation, and neutrophil extracellular traps. Neutrophils can be divided into pro-inflammatory (N1) and anti-inflammatory (N2) cells; depending on the type of cytokines they produce (Jorch and Kubes, 2017).

Eosinophils are present in the circulation and are also abundant in skin and mucosal membranes. These cells are sensitive to immune complexes in which IgE is involved. When eosinophils are in contact with these IgE-immune complexes, they degranulate and secrete cytotoxic substances, mainly against extracellular parasites (Yang et al., 2017).

Basophils are another essential, but less studied, type of granular cell. Nevertheless, it is known that they have an important role in the recruitment of leucocytes, tissue remodeling, and angiogenesis. Additionally, they produce histamine, leukotrienes, and IL-4. Another type of immune cell arises from the same progenitor as basophils: the mast cells. These cells are also called “primed cells” and are abundant in the skin, intestine, and airways, where they are involved in inflammation processes and hypersensitivity (Otsuka et al., 2016).

Macrophages and monocytes are mononuclear phagocytic cells present in tissues and in the circulation, respectively. Both have a fundamental role in inflammatory processes. Just like neutrophils, macrophages and monocytes exist as sub-populations characterized by the type of anti-inflammatory cytokine they produce and also participate in immune response regulation (Gordon and Martinez-Pomares, 2017).

Dendritic cells (DCs) are the most specialized antigen presenting cells. They are able to induce immune responses against invading pathogens. On the other hand, DCs can induce tolerance to self-antigens and exert regulatory effects on T and B cells (Goyvaerts and Breckpot, 2015).

There has been a recent interest in studying ILC populations, which are phenotypically similar to natural killer cells (NK) but, unlike these, they do not have a clear cytotoxic function. Rather, ILCs participate in regulation and cellular cooperation similarly to T helper lymphocytes (Th) during the classical adaptive immune response (Wenink et al., 2017). NK cells are the most studied type of innate lymphoid cell. Their cytotoxic activity is directed against transformed and virus-infected cells. NK cells express membrane receptors (KIR and CD94/NKG2A) that recognize HLA molecules and inhibit cytotoxic activity. In this way, when the ligands for these receptors are absent from the target cells, NK cells secrete cytokines, granzymes, and perforins that lyse transformed or infected cells (Lam and Lanier, 2016).

4. Influence of the cholinergic system on innate immune function

In essence, immune cells are endowed with the critical elements to constitute an independent cholinergic system, such as ChAT and AChE in addition to the nAChRs and mAChRs. In line with this, the presence of a non-neuronal cholinergic system in innate cells is becoming more evident. Data also point out that ACh produced by immune cells exerts immunoregulatory effects, for instance on cytokine production (Fujii et al., 2017).

4.1. Neutrophil cholinergic system

4.1.1. Synthesis, secretion and degradation of acetylcholine

Both neutrophils isolated from the blood and sputum of healthy donors and patients with chronic obstructive pulmonary disease (COPD) express ChAT and VAChT but not CHT, showing overexpression of these elements in COPD patients (Milara et al., 2016). Additionally, human epidermal neutrophils express two isoforms of ChAT-like proteins, one monomer of 54 kDa and a dimer of 69 kDa, the last one is similar to the neuronal ChAT protein (69-KDa), thus indicating that neutrophils have the biochemical machinery to produce ACh (Hagforsen et al., 2000). In agreement with this, antigenic stimulation increases ACh synthesis on immune cells, thus neutrophil cholinergic machinery could be increased on inflammatory processes (Fujii et al., 2003).

4.1.2. Cholinergic receptor expression

Regarding cholinergic receptors, there is clear evidence for the presence of nAChR on neutrophils. In this sense, C57BL/6J mice neutrophils from bone marrow ($CD11b^+Ly6G^+$) express the α6, α9, β2, β3 and β4 subunits, while α3, α4 and α7 are absent. The authors suggest that the expression of these receptors increases as immune cells progress in their development (St-Pierre et al., 2016). The evidence of the presence of nAChR on neutrophils demonstrated that these cells respond (IL-8 production) to nicotinic agonists or antagonists. Particularly, the presence of α1, α3, β2 and β4 in peripheral blood neutrophils from healthy donors has been reported. The neutrophils were unresponsive to agonists or antagonists for α4 and α7, which suggest the absence of these subunits in these cells (Iho et al., 2003). Furthermore, peritoneal neutrophils from male BALB/c mice expressed α2, α3, α4, α5, α6, α7, α9, β2, β3, and β4 subunits. The authors point to a direct participation of nAChRs in neutrophil reactions (Safronova et al., 2016). Moving onto a pathophysiological scenario, it was reported that neutrophils ($Gr1^+$) from CD1 mice express α7, and that this receptor is overexpressed in induced acute lung inflammatory injury (Su et al., 2010).

With respect to mAChR, it has been known for more than 30 years that human peripheral blood neutrophils have binding sites for agonists like oxotremorine and pilocarpine, and antagonists such as [3H]-Quinuclidinyl benzilate ([3H]-QNB) and atropine, demonstrating the presence of mAChR (Dulis et al., 1979). There are some studies that show that these cells have a differential expression pattern of these receptors in pathological conditions. In this context, peripheral blood neutrophils from healthy donors and patients with rheumatoid arthritis express the M3, M4, and M5 subtypes, but not M1 and M2. In contrast, neutrophils isolated from synovial fluid of patients express M3 and M5 but not M4 (Bany et al., 1999). Blood and sputum neutrophils isolated from COPD patients express M2, M3, M4, and M5, while M1 is absent, showing overexpression of the M2, M4 and M5 subunits of the mAChR on COPD exacerbated patients (Milara et al., 2016). Similar results were obtained with neutrophils from the sputum of COPD patients, healthy non-smoker and smoker donors, which expressed the M1, M2 and M3 subtypes. In this study, M1 and M3 expression was higher in COPD patients and smokers (Profita et al., 2005). It could be suggested that the ACh stimulation of nAChR and mAChR can generate a pro-inflammatory cytokine response and increase neutrophil recruitment mediated by chemokine over-expression (CCL-5; IL-8) in chronic inflammatory diseases such as COPD and rheumatoid arthritis (Bany et al., 1999; Hagforsen et al., 2000; Iho et al., 2003; Milara et al., 2016).

4.1.3. Cholinergic regulation of neutrophils

Regarding the cholinergic regulation of neutrophil functions, it has been observed that incubation with ACh (100 μM) significantly increases the leukotriene-B4 (LTB_4) production and chemotactic activity of neutrophils from COPD patients (Profita et al., 2005).

The neutrophil functional capacity could be altered by ACh receptor agonists and antagonists. In this sense, nicotine stimuli (0.1–10 mM) increased IL-8 neutrophil synthesis, which potentiated chemotaxis (0.6 mM), an effect that is associated with the increased H_2O_2 and O_2^- production at 2.5 and 3 mM nicotine, respectively, with the subsequent activation of the NFkB pathway (Iho et al., 2003). Nicotine (1–10 μM) decreased the expression of CD11a, CD11b, and L-selectin (CD62L). These results suggest a loss of neutrophil adhesion ability in endothelial cells and also a disturbed extravasation of neutrophils to the inflammation sites (Speer et al., 2002).

Nicotine has the capacity to alter neutrophil chemotaxis when cells are stimulated by chemotactic peptides. In this context, neutrophils

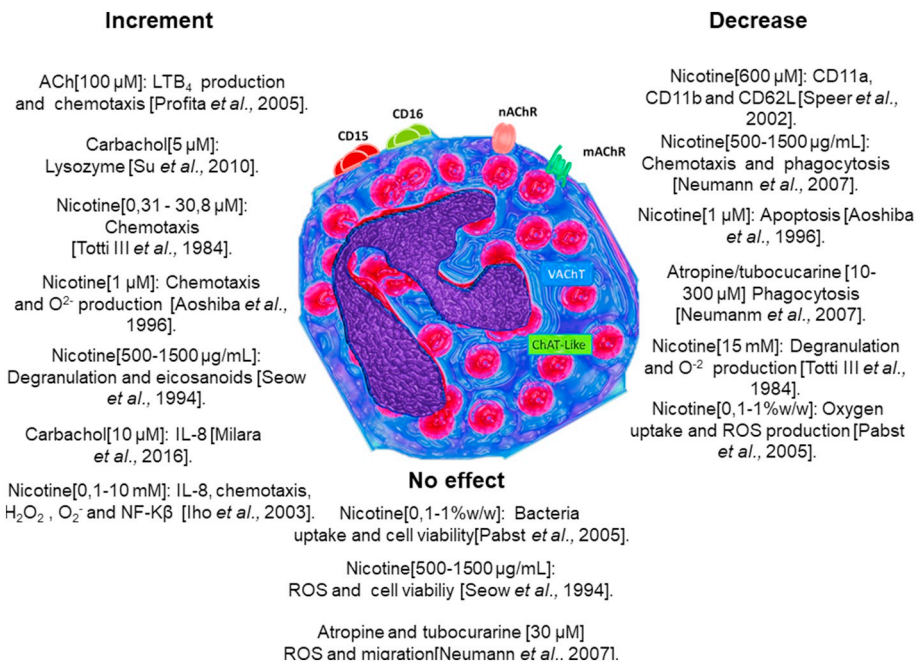

Fig. 2. Cholinergic components and influence of cholinergic agents on neutrophil function; ChAT-Like: choline acetyltransferase-type protein, CD16: low affinity Fc receptor; CD15: 3-fucosyl-*N*-acetyl-lactosamine; nAChR: nicotinic acetylcholine receptor (α1, α2, α3, α4, α5, α6, α7, α9, β2, β3, and β4) and mAChR: muscarinic acetylcholine receptor (M1-M5). Note: the elements shown in this figure are conditioned to the health status, biological model or disease in which they were described.

isolated from healthy non-smoker donors, stimulated with N-formylmethionyl-leucyl-phenylalanine (fMLP) (10^{-8} M) and exposed to 0.31 and 30.8 µM nicotine, have an enhanced chemotaxis; whereas a concentration of 15 mM of nicotine restricted lysozyme degranulation, myeloperoxidase, elastase and superoxide (O_2^-) production (Totti et al., 1984). Further, Aoshiba et al., (Aoshiba et al., 1996) reported an increase of the chemotactic activity and O_2^- production in blood neutrophils isolated from healthy, non-smoking, volunteers, stimulated with fMLP (0.1 µM) and incubated with 1 µM nicotine. Additionally, they observed an increase in neutrophil survival by the suppression of apoptosis.

On the other hand, opposite effects have been reported on neutrophils exposed to diverse chemotactic stimuli (C5a, leukotriene-β4, IL-8 and fMLP), nicotine at 1000 and 1500 µg/mL decreased the neutrophil chemotactic capacity, while at 500–1500 µg/mL it reduced the phagocytic capacity and induced degranulation of lysozyme, β-glucuronidase, elastase, prostaglandin (PGE_2), and leukotriene-C4 (LTC_4). In contrast, at the same concentration (500–1500 µg/mL) ROS production and cell viability were not affected (Seow et al., 1994). In agreement with this, alterations on LPS-primed neutrophils incubated with nicotine (0.1–1% of nicotine by dry weight from tobacco) have been reported, showing a dose-dependent decline in oxygen uptake and the production of O_2^- and H_2O_2. However, nicotine did not alter bacteria uptake capacity or neutrophil viability. This indicates that nicotine stimuli can reduce the oxygen-dependent microbicide mechanism (Pabst et al., 1995). The results above mentioned might seem contradictory given that nicotine can promote or reduce the inflammatory process in a dose-dependent manner. However, clinical evidence generally suggests that nicotine treatment could promote the inflammatory response and that it could be exacerbated in smokers (Totti 3rd. et al., 1984).

The role of a nicotine modulator has been demonstrated on neutrophil essential functions such as chemotaxis, phagocytosis, degranulation, and production of ROS, which are implicated in the generation of a pro-inflammatory microenvironment. Nonetheless, nicotine receptors have the ability to induce anti-inflammatory cytokines. Using α7 *knockout* (KO) mice treated with a subcutaneous dose of a pro-inflammatory agent (croton oil), an increase in neutrophil ($Ly6G^+$) infiltration, mRNA levels of cytokines (IL-1β and IL-6, but not TNF-α), chemokine receptor (CCR10), and adhesion molecule transcripts (ICAM-1and itga3) were observed, when compared to wild-type (WT) mice (Gahring et al., 2010). In agreement with this, neutrophils from CD1 mice incubated with nAChR α7 agonist have a significantly lower production of TNF-α and MIP-2, while CD1 nAChR $\alpha7^{+/+}$ exerted this anti-inflammatory effect (Su et al., 2010). The nAChR play an imperative role in the neutrophil-mediated immune response, the anti-inflammatory influence of this nicotinic receptor (α7) is unmistakable.

On the other hand, muscarinic cholinergic agents, similarly to nicotinic agents, can induce changes in the immune response, but there is still little information on the mAChR-mediated regulation of neutrophils. Regarding the regulatory capacity of the mAChR, when the cells were treated with atropine (1 µM), spontaneous migration and generation of ROS and phagocytosis were not modified, however, when the cells were treated with tubocurarine, a significant decrease in phagocytic capacity was observed, This effect was enhanced when atropine was added (Neumann et al., 2007). Further, the mAChR and nAChR agonist carbachol (5 µM) increases lysozyme release in neutrophils ($Gr1^+$ cells) (Su et al., 2010), and carbachol at 10 µM induced IL-8 release in isolated blood and sputum neutrophils from healthy donors and COPD patients (Milara et al., 2016).

Agonists and antagonists of the cholinergic receptors in neutrophils have an evident effect in upregulating or downregulating innate responses. These phenomena impact on the migration, phagocytic capability, degranulation, ROS generation, cytokine production, and expression of adhesion molecules, leading to substantial modifications in the neutrophil ability to cope with microbial infections and the accompanying inflammatory response. A summary of the reported effects of the cholinergic system on neutrophils is provided in Fig. 2.

4.2. Eosinophil cholinergic system

4.2.1. Synthesis, secretion and degradation of acetylcholine

Until now, the information reported suggests that blood eosinophils from healthy donors do not express ChAT, VAChT or AChE, which are essential proteins for the synthesis, storage and degradation of ACh, respectively (Durcan et al., 2006). However, eosinophils isolated from the skin of patients with palmoplantar pustulosis expressed low levels of ChAT-like proteins (54 KDa). Experimental results suggest that during normal conditions eosinophils do not produce ACh, but are able to do it under a pathophysiological scenario, maybe as part of an anti-inflammatory regulatory mechanism (Hagforsen et al., 2000).

4.2.2. Cholinergic receptor expression

With respect to nAChR, blood eosinophils isolated from mild asthmatic non-smokers express α3, α4 and α7 subunits (Blanchet et al., 2007). In line with this, eosinophils from patients with recurrent allergies express the α4, α7 and the α1/α3/α5 subunits (Watson et al., 2014). The results from these studies suggest that eosinophils from patients with inflammatory processes express a set of nAChR.

Regarding mAChR expression, the expression of the M2 subunit was analyzed in blood eosinophils from healthy donors, which did not express the M2 subtype (Durcan et al., 2006). Similarly, eosinophils from the blood of healthy female Dunkin-Hartley guinea pigs did not express M2, M1 or M5; however, M3 and M4 expression was detected (Verbout et al., 2009). Nevertheless, in a pathologic inflammatory situation such as ulcerative colitis, eosinophils from human blood and colon mucosa show an increased expression of M1, M2, and M3 subtypes, compared to healthy subjects (Wallon et al., 2011). On the other hand, mAChR expression of M1, M2, and M5 has been detected on the human eosinophil cell line 15HL-60 (Wallon et al., 2011). In contrast, the expression pattern of mAChR subtypes was evaluated on the eosinophilic leukemia cell line EoL-1, showing no expression for M1, M2, M3, M4 and M5 (Mita et al., 1996).

4.2.3. Cholinergic regulation of eosinophils

Cholinergic regulation is important in pathological processes; therefore, it is essential to study the anti-inflammatory phenomenon. Blanchet et al., (Blanchet et al., 2007), reported the effect of dimethylphenylpiperazinium (DMPP), a non-selective nAChR agonist, which at 80–360 μM reduces LTC_4 secretion on eosinophils stimulated with platelet-activating factor (PAF) (1 μM) and C5a (0.1 μM). On the other hand, DMPP at 160 μM reduces PAF-induced intracellular calcium mobilization, the expression of matrix metallopeptidase 9, as well as eotaxin or 5-Oxo-ETE-induced eosinophil migration, with no effect on eosinophil viability. These anti-inflammatory effects on migration were reversed by treatment with mecamylamine, a non-selective antagonist of nAChR. Taken together, these results indicate that functional nAChRs are expressed in eosinophils and that nAChR agonists down-regulate eosinophil function in vitro. The inhibitory magnitude of the nicotinic agonist on eosinophil responses put forward a potential pharmacologic treatment of chronic diseases such as asthma (Blanchet et al., 2007). Further, an increase in eosinophil activation in guinea pigs treated with 1 mg/Kg atropine has been reported (Verbout et al., 2009). A summary of the modulation of eosinophils by the cholinergic system is provided in Fig. 3.

Eosinophils constitute an important, previously missing, link in the signaling events. Potentially, these neuroimmune events may be important as an integrated part of stress-related disease flares (Wallon et al., 2011). nAChR and mAChR are expressed in eosinophils. Since eosinophils are essential for host defense against extracellular parasites, the question remains as to whether nicotinic agonist exposure may interfere with the eosinophil defense mechanisms (e.g., secretion of cytotoxic substances and migration). This issue may become even more relevant in people from underdeveloped countries with smoking habits and the potential detrimental influence on the rate and spread of extracellular parasites.

4.3. Basophil cholinergic system

4.3.1. Synthesis, secretion and degradation of acetylcholine

There are no studies showing the expression of proteins for the synthesis, transport, and degradation of ACh in basophils. Nevertheless it has been observed that basophils respond to cholinergic agents (Thompson-Cree et al., 2004; Mishra et al., 2010; Watson et al., 2014) suggesting the presence of cholinergic receptors.

4.3.2. Cholinergic receptor expression

Regarding nAChR, blood basophils from subjects with mild allergic asthma express the α4, α7 and the α1/α3/α5 subunits (Watson et al., 2014). nAChR expression has also been reported in basophil cell lines. In this context, the presence of the α7 subunit gene and transcript has been evidenced in the human basophil cell line KU812 (Sudheer et al., 2006). Up to now, information on the cholinergic system of basophils is scarce. Currently there are only rough reports in reference to nicotinic receptors, so it is essential to expand the study of these elements since they could play an important role in hypersensitivity reactions.

4.3.3. Cholinergic regulation of basophils

Cholinergic agents like nicotine alter basophil functions. This has been demonstrated in smokers, who had an increased peripheral basophil number and degranulation (Jensen et al., 1998; Walter and Walter, 1982). In line with this, the nicotine isomers [−]-1-methyl-2-[3-pyridyl] pyrrolidine (1 mM) and (+)-Nicotine (+)-di-*p*-toluoyltartrate salt (0.01 and 1 mM), decreased IgE-mediated histamine release in basophils from healthy donors, whereas cell viability was not altered (Thompson-Cree et al., 2004). Similar effects of histamine release were obtained in blood basophils from mild allergic asthma patients incubated with the nicotinic agonist ASM-024 (0.01 mM, 0.1 mM, and 1 mM), whereas a decrease in CD203c was detected in basophils incubated with 0.1 mM of this agonist. In this study, Ipratropium (muscarinic antagonist), hexamethonium (nicotinic antagonist) and α-bungarotoxin reverted this effect. The above demonstrates that these cells are capable of responding to the muscarinic antagonist, although until now there was no evidence of expression. This could suggest the presence of these receptors in basophils and it can be speculated that they also express muscarinic receptors (Watson et al., 2014). The reported effects of cholinergic system modulation on basophils are shown in Fig. 4.

4.4. Mast cell cholinergic system

4.4.1. Synthesis, secretion, and degradation of acetylcholine

In regard to ACh synthesis, ChAT immunoreactivity has been reported in skin mast cells from patients with atopic dermatitis. Additionally, in skin biopsy samples from healthy donors, ChAT activity and ACh concentration were lower compared with patients with atopic dermatitis (non-eczematous). In patients with eczematous atopic dermatitis, the ACh concentration was exacerbated 4 and 14 fold, respectively (Wessler et al., 2003). However, ChAT expression was not detected in skin mast cells of healthy volunteers or in patients with palmoplantar pustulosis (Hagforsen et al., 2000). In spite of the fact that the neutrophils, basophils, and eosinophils do not express AChE, human and murine mast cell lines (HMC-1 and MC-9) express AChE (Nechushtan et al., 1996). Additionally, skin mast cells of patients with palmoplantar pustulosis displayed higher AChE immunoreactivity when compared to healthy volunteers or smokers (Hagforsen et al., 2000). This suggests that mast cells are able to synthesize and degrade ACh in some pathological skin conditions and could contribute to the pathogenesis of inflammatory skin diseases.

4.4.2. Cholinergic receptor expression

Cholinergic receptor expression has been proved in several studies. Specifically, differentiated mucosal-type mast cells derived from male BALB/c mice bone marrow expressed the α4, α7 and β2 subunits, the latter exhibited higher expression, while the α3 subunit was not detected (Kageyama-Yahara et al., 2008). This indicates that mast cells might express nAChR constitutively. Nevertheless, nAChR subunit expression could be different during inflammatory processes. In line with this, skin mast cells from healthy donors and patients with atopic dermatitis expressed the α10 subunit; while mast cells of the lesioned skin area of patients with atopic dermatitis also expressed the α3 and α5 subunits (Masini et al., 1983). Additionally, expression of the α7 subunit has been demonstrated in the human mast cell line HMC-1 (Sudheer et al., 2006).

In regard to mAChR, research groups have demonstrated the presence of mAChR in mammalian mast cells using agonists and/or antagonists (Masini et al., 1983; Nemmar et al., 1999). In particular, the mAChR M2 was identified in intraperitoneal rat mast cells through the

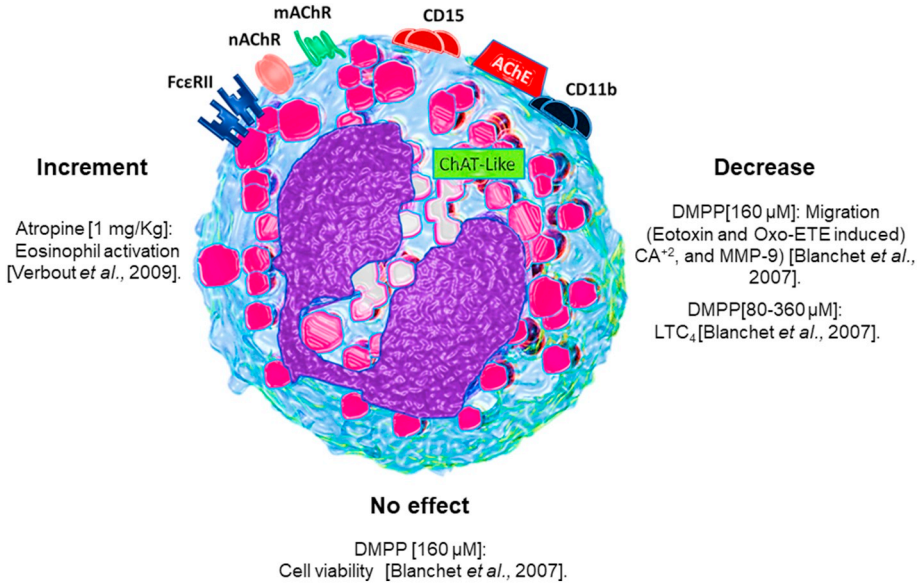

Fig. 3. Cholinergic components and influence of cholinergic agents on eosinophil function. ChAT-Like: choline acetyltransferase-type protein; AChE: Acetylcholinesterase; CD15: 3-fucosyl-*N*-acetyl-lactosamine; CD11b: integrin αM; nAChR: nicotinic acetylcholine receptor (α1, α3, α4, α5, and α7) and mAChR: muscarinic acetylcholine receptor (M1-M5). Note: the elements shown in this figure are conditioned to the health status, biological model or disease in which they were described.

selective antagonist methoctramine (Chahdi et al., 1998). From this, it can be speculated that mAChRs are expressed in mast cells.

4.4.3. Cholinergic regulation of mast cells

Due to the fact that mast cells could express cholinergic receptors, these kinds of agents may regulate mast cell function. In this regard, intraperitoneal mast cells from rats exposed to ACh (10^{-13} and 10^{-9} M) had an increased histamine release, an effect reversed with atropine (Fantozzi et al., 1978). In this sense, the selective M2 antagonist methoctramine (3 μM-1 mM) induced histamine release in intraperitoneal rat mast cells, an effect reversed by G protein antagonists. This effect could be attributed to the fact that when mAChRs are stimulated, intracellular signaling pathways mediated by G proteins are activated, so the use of antagonists will block the signaling mediated by G proteins and therefore the mAChR activity (Chahdi et al., 1998). On the other hand, it was found that the antigen-induced β-hexosaminidase degranulation on differentiated mucosal-type mast cells derived from male BALB/c mice bone marrow is inhibited by treatment with nicotine (3.2 mM), epibatidine (3.2 mM), ACh (10 mM), and GTS-21 (100 μM). GTS-21 was more effective than the other agonists; however the GTS-21-evoked inhibitory effect on degranulation was reversed with α-bungarotoxin (Kageyama-Yahara et al., 2008). Although cholinergic regulation mediated by mAChR is essential in immune cells, the information published so far is insufficient. A summary of the reported effects of the cholinergic system on mast cells can be seen in Fig. 5.

Basophils and mast cells allow the recruitment of immune cells and send potent alarm signals that play a role in hypersensitivity reactions. Hence, it is essential to properly regulate these mechanisms. The downregulation of cholinergic effects on histamine and β-hexosaminidase release along with the ameliorated inflammation provide a strong basis for the therapeutic use of cholinergic compounds in allergic pathological states like asthma.

4.5. Monocytes

4.5.1. Synthesis, secretion, and degradation of acetylcholine

ACh synthesis, storage, and degradation have been demonstrated in monocytes. According to this, studies in the human monocyte cell line U937 reported the presence of elements for ACh synthesis and degradation (ChAT and AChE, respectively), as well as the concentrations

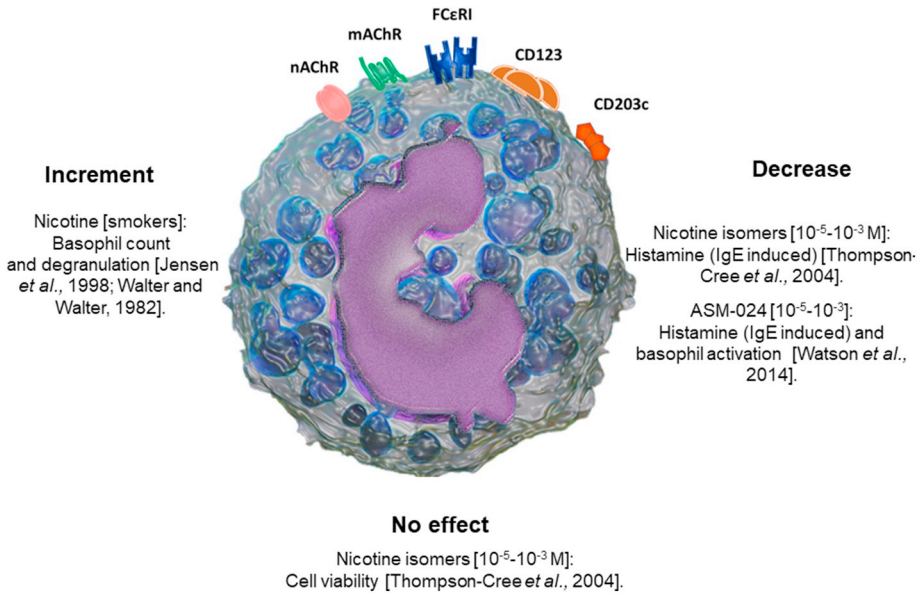

Fig. 4. Cholinergic components and influence of cholinergic agents on basophil function. CD123: a-chain of the IL-3 receptor; CD203c: basophil-specific ectoenzyme *E*-NPP3; FCεRI: IgE Receptor; nAChR: nicotinic acetylcholine receptor (α1, α3, α4, α5, and α7) and mAChR muscarinic acetylcholine receptor. Note: the elements shown in this figure are conditioned to the health status, biological model or disease in which they were described.

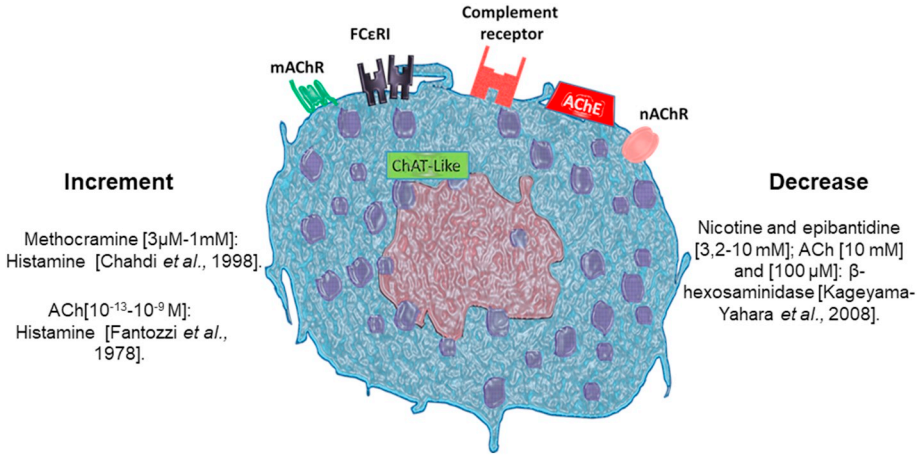

Fig. 5. Cholinergic components and influence of cholinergic agents on mast cell function. ChAT-Like: Choline acetyltransferase-type protein; AChE: Acetylcholinesterase; FCεRI: IgE Receptors; nAChR: nicotinic acetylcholine receptor (α3, α4, α5, α7, and α10) and mAChR muscarinic acetylcholine receptor (M2). Note: the elements shown in this figure are conditioned to the health status, biological model or disease in which they were described.

of ACh (0.02 ± 0.01 pmol/10^6 cells) (Kawashima and Fujii, 2000; Kawashima and Fujii, 2003). In agreement with this, rat monocytes express pChAT (Hecker et al., 2006). Also, the presence of cholinesterases in human blood monocytes has been suggested (Whaley et al., 1981). Within the setting of the extraneuronal system, it was found that activated monocytes present in blood vessels of rat renal grafts increased the ACh synthesis during acute rejection (Hecker et al., 2009; Wilczynska et al., 2011). From these data it can be suggested that monocytes can synthesize and degrade ACh. Similarly to other immune cells, ACh production is increased during pathological conditions. Other reports have pointed out that this phenomenon is important in allograft rejection.

4.5.2. Cholinergic receptor expression

In regard to nAChR expression, monocytes isolated from the bone marrow of C57BL/6J mice express the α6, α7, α9, β2, β3, and β4 subunits (St-Pierre et al., 2016). Additionally, nAChR has been detected in human peripheral blood monocytes by using nicotine-[^{3}H], tubocurarine, and α-bungarotoxin (Whaley et al., 1981; Davies et al., 1982). In line with this, human and rat monocytes express the α7, α9, and/or α10 subunits (Hecker et al., 2015); while human monocyte cell lines (THP-1, U937, and Mono-Mac-6), as well as human monocytes from healthy donors, express the α7 subunit (Wang et al., 2003; Yoshikawa et al., 2006; van der Zanden et al., 2012; Benfante et al., 2011). This may indicate that a set of nAChR is expressed constitutively on monocytes, even during early cell development (St-Pierre et al., 2016).

The reported results on the presence of mAChR in monocytes are contradictory. The absence of muscarinic receptor mRNA in human monocytes (M3, M4, and M5) was reported (Hellström-Lindahl and Nordber, 1996). Owing to the fact that these cells did not respond to treatment with muscarinic antagonists (atropine and [^{3}H]-*n*-methylscopolamine), it was suggested that monocytes do not express mAChR (Whaley et al., 1981; Eva et al., 1989). However, human monocytes have affinity for [^{3}H]-QNB, suggesting the presence of mAChR (Eva et al., 1989). In contrast, Pahl et al., 2006, reported the presence of M1, M3, M4, and possibly M2 mRNA, and the absence of M5, in monocytes from healthy donors. Additionally, M3 is expressed in healthy human monocytes (Profita et al., 2005). Therefore, it is necessary to perform more studies in monocytes.

4.5.3. Cholinergic regulation of monocytes

The cholinergic regulation of monocytes has been demonstrated for more than 30 years. In this context, ACh, as well as carbachol (10^{-8} M-10^{-4} M), but not pilocarpine (mAChR agonist) (10^{-8}–10^{-4} M), stimulated the secretion of component 2 (C2) from complement system in peripheral blood monocytes (Whaley et al., 1981). Furthermore, ACh (100 μM) stimulated the ERK1/2 pathway and LTB_4 production in monocytes from peripheral blood of healthy volunteers; and this effect was blocked by a pre-treatment with the mAChR antagonist oxitropium bromide (10 μM) (Profita et al., 2005).

The anti-inflammatory effect of the nicotinic agonist on monocytes is evident. In line with this, evidence supports the inhibiting effect of nicotine (1% dry weight) on O_2^- and IL-1β production in LPS-stimulated (1 ng/mL) monocytes (Pabst et al., 1995). Further, in LPS-stimulated (3 mg/mL) human monocytes, nicotine (10^{-14}–10^{-11} M) reduced TNF-α production (van der Zanden et al., 2012; Brampton et al., 2000). Likewise, in peripheral blood monocytes exposed to 10^{-8} M nicotine, TNF-α and inflammatory macrophage protein 1-α (MIP-1-α) production was reduced (Davies et al., 1982). There is evidence of the importance of α7 in monocyte inflammatory response regulation. For example, using antisense oligonucleotides for α7, the authors demonstrated the inhibitory effect of nicotine on TNF-α production (Wang et al., 2003).

The metabotropic function of the nAChR has been demonstrated. A study in primary rat monocytes and alveolar macrophages demonstrated that the metabotropic activity of nAChR in these cells reduced ATP-induced calcium signals (Mikulski et al., 2010), implying some sort of interference with the ATP/P2X7 receptor signaling, which activates the NLRP3-containing inflammasome and induces the release of mature IL-1β (Gross et al., 2011; Rathinam et al., 2012). In line with this, ACh, choline, phosphocholine (PC), phosphocholine-modified LPS from *Haemophilus influenzae*, and phosphocholine-modified protein, efficiently inhibited ATP-mediated IL-1β release in human and rat monocytes via subunits α7, α9, and/or α10 from nAChR (Hecker et al., 2015). This PC-mediated inhibition was not accompanied by a stimulation of the canonical ionotropic receptor at canonical human α9 nAChR homopentamer and α9/α10 nAChR heteropentamers; rendering PC a potential therapeutic anti-inflammatory devoid of adverse ionotropic activity of excitable cells (Richter et al., 2016). In a more recent study on peripheral blood mononuclear cells, the authors further demonstrated that subunits α7, α9 and α10 were involved in the inhibition of the ATP-dependent release of IL-1β by ACh, nicotine, and PC. Lysophosphatidylcholine (LPC) and glycerophosphocholine (G-PC) also inhibited ATP-dependent release of IL-1β, although the LPC inhibitory effect was mostly mediated by the α9 and α10 subunits of the nAChR. In essence, canonical nicotinic agonists and PC elicit metabotropic nAChR activity in monocytes via interaction of subunits α7, α9 and α10 of the nAChR; whereas the metabotropic signaling of LPC and G-PC, rely mostly on the α9 and α10 subunits of nAChR (Zakrzewicz et al., 2017). The reported effects of the cholinergic system on monocyte function are provided in Fig. 6.

4.6. Macrophages

4.6.1. Synthesis, secretion, and degradation of acetylcholine

Macrophages have the biochemical machinery to synthesize ACh. Specifically, it has been demonstrated that alveolar macrophages

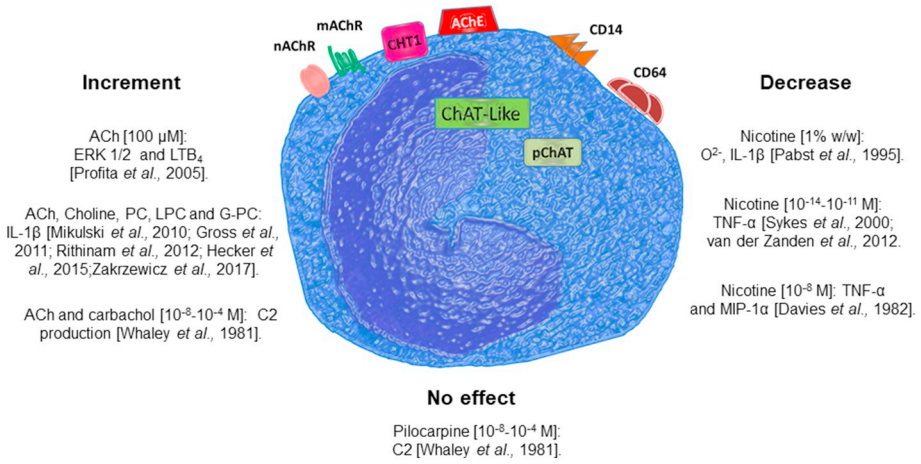

Fig. 6. Cholinergic components and influence of cholinergic agents on monocyte function. pChAT: Peripheral choline acetyltransferase; AChE: Acetylcholinesterase; CHT1: High affinity choline transporter; CD14: TLR4 co-receptor; CD64: Fc fragment of IgG, high affinity Ia receptor; nAChR: nicotinic acetylcholine receptor (α6, α7, α9, α10, β2, β3, and β4) and mAChR: muscarinic acetylcholine receptor (M1-M4). Note: the elements shown in this figure are conditioned to the health status, biological model or disease in which they were described.

express ChAT (Wessler and Kirkpatrick, 2001). Additionally, ChAT-GPF (ChAT coupled to green protein fluorescent) expression was also demonstrated in mice macrophages ($CD11b^+CD11c^-F4/80^+$) (Reardon et al., 2013). In agreement with this, lung and alveolar macrophages express ChAT and also VAChT and CHT1 (Koarai et al., 2012). Furthermore, macrophages isolated from C57BL/6J mice, as well as the mouse macrophage cell lines NR8383 and CD1 express AChE (Kawashima et al., 2007; Su et al., 2010; Liu et al., 2015). These data suggest that macrophages express proteins for the synthesis, transport, and degradation of ACh.

4.6.2. Cholinergic receptor expression

In addition to having the biochemical elements to synthesize and degrade ACh, macrophages, like other immune cells, express cholinergic receptors. The presence of α1, α7, and α10 mRNA, as well as the α7 subunit protein, has been reported on human blood differentiated macrophages (Wang et al., 2003).

Additionally, murine microglia, peripheral macrophages of C57BL/6 mice, as well as CD1 mice alveolar macrophages, express the α7 subunit (Shytle et al., 2004; Su et al., 2010). Furthermore, spleen macrophages from C57BL/6J mice express the α2, α4, α5, α6, α7, α10, β2, and β4 subunits (Kawashima et al., 2007). Moreover, α7 expression but not α9 was demonstrated in spleen and head kidney derived macrophages from a teleost fish (rainbow trout). Similarly to mammalian immune cells, α7 was increased in trout macrophages when these cells were poly I:C and LPS-stimulated (Torrealba et al., 2018). The α4 and β2, but not α7, subunit expression was reported in the mouse alveolar macrophage cell line (MH-S) (Matsunaga et al., 2001), while the monocyte/macrophage cell line (J774A.1) expressed the nicotinic receptor formed by subunits α4β2 (Kiguchi et al., 2015).

Several authors have evidenced that mouse and human macrophages express mAChR (M1-M5) (Kawashima et al., 2007; de la Torre et al., 2008; Koarai et al., 2012). Moreover, in a pathological scenario, mAChR expression has been proved (M2 and M3) on human macrophages isolated from sputum (Gwilt et al., 2007; Profita et al., 2005). Additionally, the expression of mAChR has been demonstrated in other mammals; specifically, bovine alveolar macrophages express M3 (Sato et al., 1998). This indicates that macrophages constitutively express a set of nAChR and mAChR receptors.

4.6.3. Cholinergic regulation of macrophages

ACh exerts an anti-inflammatory effect on macrophages, similarly to what has been demonstrated in monocytes. When differentiated macrophages derived from healthy donor blood monocytes are incubated with ACh (0.001–100 μM), they secrete significantly lower quantities of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-18), but the anti-inflammatory cytokine IL-10 was not altered (Borovikova et al., 2000). Furthermore, in intestinal and peritoneal macrophages from BALB/c and C57/Bl6 mice, ACh (1 μM) increased IL-10 production. In line with, ACh (10^{-7}–10^{-6} M) reduced NF-KB activity, TNF-α production and the expression of the macrophage chemotactic protein MCP-1. In addition to this anti-inflammatory effect, intestinal macrophages isolated from C57/B16 male mice incubated with ACh (10^{-6} M) and nicotine (10^{-8}–10^{-6} M), exhibited a significant increase in phagocytosis, mainly induced by nAChR α4β2, rather than nAChR α7. This effect was mediated by the stimulated recruitment of the dynamin-2 GTPase into the forming phagocytic cup (van der Zanden et al., 2009).

The effect of ACh on the modulation of inflammation could be similar to that exerted by nicotinic agonists. In line with this, alveolar macrophages from CD1 mice incubated with nicotine (1 μM), had a significantly lower production of TNF-α and MIP-2, while incubation with the α7-antagonist methyllycaconitine (MLA) at 1 μM inhibited this anti-inflammatory effect (Su et al., 2010). That effect also has been reported in murine alveolar macrophages infected with the bacterium *Legionella pneumophila* and exposed to nicotine (0.1, 1, and 10 μg/mL) (Matsunaga et al., 2001). In agreement with this, when the J774A.1 cell line is incubated with LPS (50 ng/mL) and the selective α4β2 agonist (TC-2559 ([(*E*)-N-Methyl-4-[3-(5-ethoxypyridin)yl]-3-buten-1-amine]); at 100 μM), IL-1β mRNA levels are reduced and at higher doses (500 μM) the C-motif chemokine ligand decreases (CCL3) (Kiguchi et al., 2015).

In particular, the role of the α7 subunit in the anti-inflammatory pathway has been evidenced by several assays. For example, α7 KO mice have significantly higher levels of pro-inflammatory cytokines (TNF, IL-1β, and IL-6) compared to WT mice (Wang et al., 2003). Pharmacological administration of α7 nAChR agonists can cause concentration-dependent inhibition of the production and release of pro-inflammatory mediators like IL-1β, TNF-α, IL-6, and the high mobility group box 1 protein (HMGB1) on macrophages, without affecting the synthesis of anti-inflammatory cytokines (Hoover, 2017). These findings on the nicotine- or ACh-induced inhibition of pro-inflammatory cytokine production may contribute to the susceptibility to infections (Matsunaga et al., 2001). When trout macrophages are treated with nicotine (100 μM) after stimulation with a TLR3 ligand poly (I:C), the expression of genes related to antiviral immunity decreases (INF-1, Mx-1, CCL4, CK6, and TLR3), but not that of genes involved in the pro-inflammatory response (TNFα, IL-6, and IL-1β). On the other hand, nicotine (100 μM) or ACh (500 μM) did not modulate phagocytosis in trout head kidney derived macrophages. Data obtained in this study showed that nAChR modulates the innate immune response on teleost fish (Torrealba et al., 2018).

Even though mAChR is essential to the regulation of the immune response, few studies have been performed on this particular topic. It has been reported that carbachol (100 μM) has a proliferative effect in these cells and induces moderate prostaglandin E2 liberation mediated by M1-M3 receptors as well as an increase on M2-induced PKC

translocation (de la Torre et al., 2008).

Monocytes and macrophages are mononuclear phagocytes playing central but different roles in tissue homeostasis and immunity. The former mostly deal with inflammation following a pathogen challenge, with tissue-resident macrophages exerting significant functions in tissue homeostasis and the resolution of inflammation. Challenging the traditional view that monocytes and macrophages arise from a continuum of differentiation, it is becoming clear that while monocytes can differentiate into macrophages, monocytes do not substantially contribute to most tissue macrophage populations. In fact, adult tissue macrophages originate from embryonic precursors colonizing tissues before birth being also able to self-renew during adult life (Ginhoux and Jung, 2017). Reviewed data point out that ACh, as well as nicotinic and cholinergic agents, reduce the LPS-induced production of pro-inflammatory cytokines (TNF-α IL-1β, IL-6, and IL-18), along with some potential effects on other anti-microbial processes such as migration, phagocytosis and ROS production (summarized in Fig. 7). It follows that in addition to its anti-inflammatory effects, the possibility that cholinergic agents may also reduce pathogen clearance by monocytes and macrophage should not be overlooked.

4.7. Dendritic cells (DCs) cholinergic system

4.7.1. Synthesis, secretion, and degradation of acetylcholine

Mouse bone marrow-derived DCs and human differentiated DCs (obtained from human monocytes cultured with IL-4 and GM-CSF) express ChAT (Kawashima et al., 2007; Salamone et al., 2011). In line with this, ChAT-GFP expression was detected in mouse splenic DCs (FSC^{hi} $CD11b^{-}CD11c^{+}MHCII^{+}F4/80^{-}$ and $CD11b^{+}CD11c^{+}MHCII^{+}F4/80^{-}$) after microbial colonization (Reardon et al., 2013). In addition, AChE expression has been demonstrated in mouse bone marrow-derived DCs and human DCs (Kawashima et al., 2007; Salamone et al., 2011). From these studies it can be suggested that DCs have the capacity to synthesize and degrade ACh. In addition, antigenic stimuli enhanced or activated the cholinergic machinery in these cells.

4.7.2. Cholinergic receptor expression

Cholinergic receptor expression has been demonstrated on DCs. Specifically, mouse DCs express the α2, α5, α6, α7, α10, β2, and β4 nAChR subunits, as well as the five mAChR subtypes (M1-M5) (Kawashima et al., 2007). Human differentiated DCs (obtained from human monocytes cultured with IL-4 and GM-CSF) express the M3, M4, and M5, but not the M1 or M2 subunits (Salamone et al., 2011). In a pathological context, nasal DCs ($CD11c^{+}$) from patients with nasal polyposis and challenged with staphylococcal enterotoxin B (SEB), had increased levels of the M3 receptor at the mRNA and protein levels (Liu et al., 2010). DCs are the main antigen presenting cells and are in direct contact with the nerve terminals in the gut-associated lymphoid tissue (GALT). In agreement with this, the M2 subtype was detected in DCs ($CD11c^{+}$) from mice Peyer's patches, demonstrating the cross-talk between these two tissues (Ma et al., 2007).

4.7.3. Cholinergic regulation of dendritic cells

Some studies provide evidence for DC regulation by cholinergic agents. For example, cholinergic stimulation with carbachol (10^{-9} M) during the differentiation of DCs induced an increase in the expression of HLA-DR and CD86 as well as on the production of TNF-α and IL-8, an effect that could be prevented with atropine. DCs treated with ACh or carbachol after differentiation improved the T cell priming ability of DCs and induced the expression of HLA-DR, and TNF-α, but not of IL-10 and IL-12. Interestingly, in LPS-primed DCs, the effect on HLA-DR and TNF-α was inhibited. Carbachol (10^{-9} M) had no effect on CD1a, CD40, CD83, and CCR7 expression (Salamone et al., 2011). These results support the existence of an autocrine/paracrine loop through which ACh modulates the function of DCs (Salamone et al., 2011). The reported effects of the cholinergic system on monocyte function are provided in Fig. 8.

Dendritic cells are the most important cells in antigen presentation and modulation of B and T cells responses. The potential impact of such cholinergic influence on DC function is not yet fully established. The effect of the cholinergic system on the antigen presentation abilities of DCs, T and B cell activation, and the development of the adaptive response are issues that should be further studied.

4.8. Innate lymphocytes cholinergic system

4.8.1. Synthesis, secretion, and degradation of acetylcholine

The presence of the non-neuronal cholinergic system in cells of adaptive immunity such as B and T lymphocytes is widely described (Benhammou et al., 2000; Tayebati et al., 2002; Kawashima and Fujii,

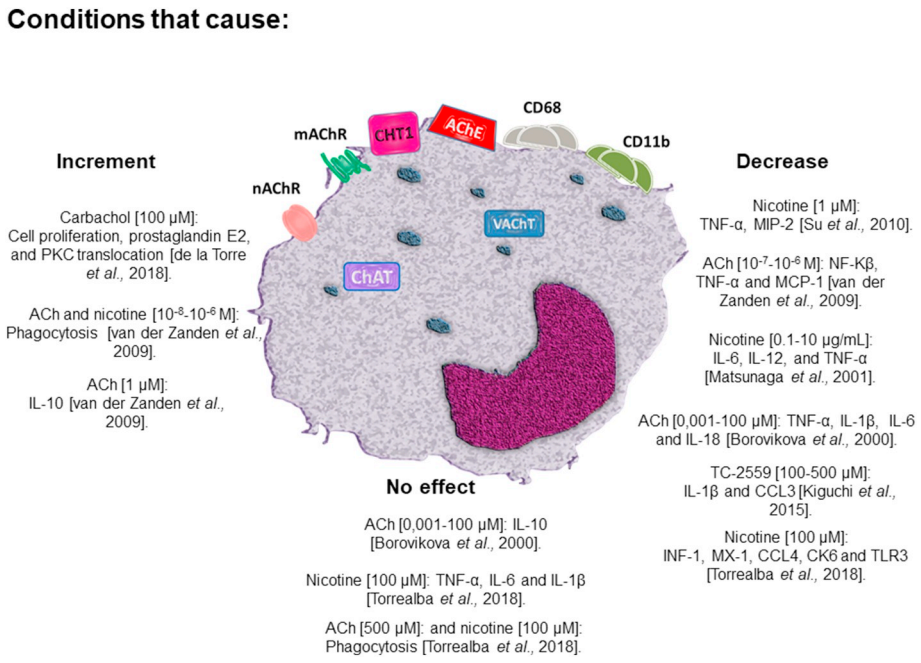

Fig. 7. Cholinergic components and influence of cholinergic agents on monocyte function. ChAT: Choline acetyltransferase; VChAT: Vesicular transporter of acetylcholine; CHT1: High affinity choline transporter; AChE: Acetylcholinesterase; CD68: glycoprotein which binds to low-density lipoprotein; CD11b: CD11b: integrin αM; nAChR: nicotinic acetylcholine receptor (α1, α2, α4, α5, α6, α7, α10, β2, and β4) and mAChR: muscarinic acetylcholine receptor (M1-M5). Note: the elements shown in this figure are conditioned to the health status, biological model or disease in which they were described.

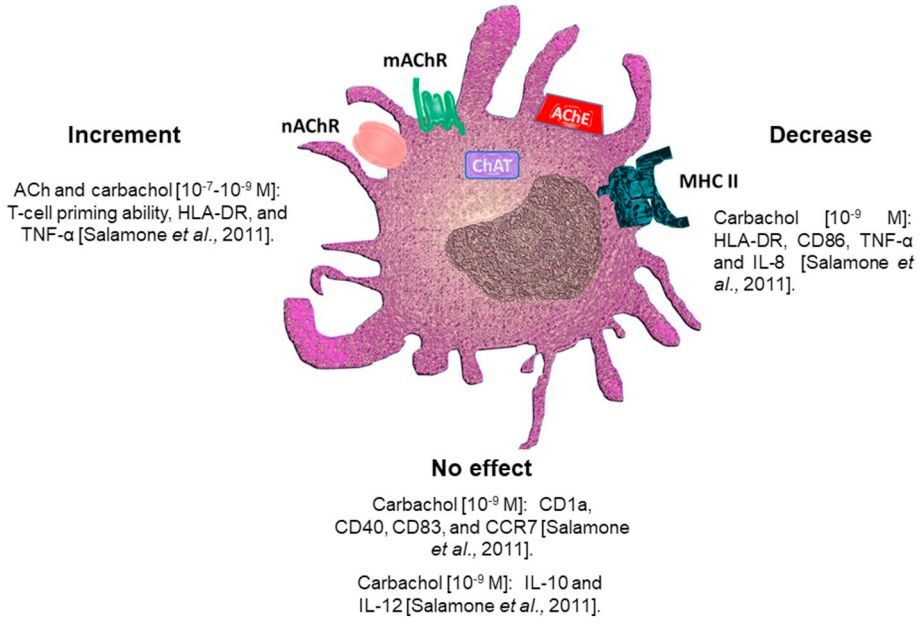

Fig. 8. Cholinergic components and influence of cholinergic agents on dendritic cell function; AChE: Acetylcholinesterase; Chat-Like: protein Choline Acetyltransferase-Like; MHC II: Major Histocompatibility Complex; nAChR: nicotinic acetylcholine receptor (α2, α5, α6, α7, β2 and β4) and mAChR muscarinic acetylcholine receptor (M1-M5). Note: the elements shown in this figure are conditioned to the health status, biological model or disease in which they were described.

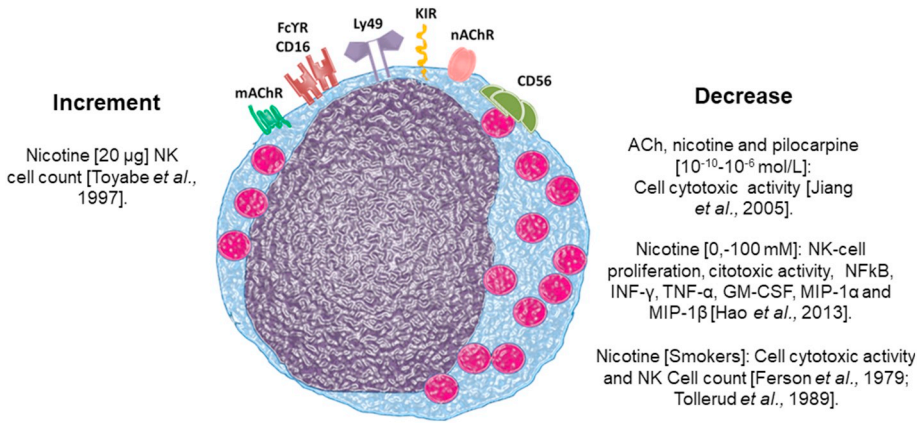

Fig. 9. Cholinergic components and influence of cholinergic agents on NK lymphocyte function. FcγR: Fc receptor; CD56: Neural cell adhesion molecule; KIR: Killer-cell immunoglobulin-like receptors; Ly49: killer cell lectin-like receptor subfamily A; nAChR: nicotinic acetylcholine receptor (α4, α5, α6, β2 and β3) and mAChR muscarinic acetylcholine receptor. Note: the elements shown in this figure are conditioned to the health status, biological model or disease in which they were described.

2003; Gwilt et al., 2007). However, the aim of this section is to refer to lymphocytes of innate immunity, but until now the existing information is scarce and the reports have only focused on NK cells. In this context, ChAT-GFP expression was not detected in NKT, TCRαβ, TCR-γδ, or NK cells (Reardon et al., 2013). Up to now, the evidence suggests that innate lymphocytes cells do not synthesize ACh.

4.8.2. Cholinergic receptor expression

Regarding cholinergic receptors, NK cells isolated from C57BL/6 female mice (RAG2$^{-/-}$) expressed the α4, α5, α6, β2 and β3, but not the α3, α7, α9 or β4 subunits (Hao et al., 2013). Additionally, murine NK cells are responsive to atropine, a mAChR antagonist (Jiang et al., 2005), which suggests that NK cells could express mAChR.

4.8.3. Cholinergic regulation of NK cells

In regards to the cholinergic regulation of NK cells, it has been reported that ACh, nicotine or pilocarpine, at 10^{-10}–10^{-6} M, inhibit the cytotoxic activity of murine spleen NK cells against the YAC-1 lymphoma cell line. Additionally, atropine blocks the inhibitory effect of ACh on NK cell cytotoxicity, but tubocurarine exerted no effect (Jiang et al., 2005).

Other studies have demonstrated that smoking leads to a decrease in circulating NK lymphocytes and their cytotoxic activity against the human melanoma cell line MM200 and hepatic Chang cells (Ferson et al., 1979; Tollerud et al., 1989). Furthermore, the NK population is significantly increased in C3H/He mice given intraperitoneal nicotine (20 μg) (Toyabe et al., 1997). In C57BL/6 female mice, nicotine (0.1–100 mM) reduced NK cell proliferation and cytotoxic activity against YAC-1 cells; as well as the production of IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), MIP-1α and MIP-1β, and the transactivation of NF-kB (Hao et al., 2013). A summary of NK cell modulation by the cholinergic system is provided in Fig. 9.

The importance of innate lymphocyte populations is still not well understood, but evidence suggest that they have and important role on innate mucosal defense. Specifically, NK cells protect us against viral infections (infected cells) and cancer (transformed cells). In this sense, AChR agonists may compromise NK cell functions, especially on lung viral infections in smoking patients. The potential contribution of the extraneuronal pathways in the development of smoking-associated cancer, through alterations in anti-neoplastic responses, should be explored carefully.

5. Conclusion

The presence of an extraneuronal cholinergic system (ACh receptors, enzymes for ACh synthesis and degradation) was first described in cells of the adaptive immune system (T- and B-lymphocytes and lymphoid cell lines). However, innate cells have an independent cholinergic system, in which ACh, as well as cholinergic agents, are quite likely to modify the inflammatory response together with innate

defense mechanisms, i.e., phagocytosis as well as ROS and cytokine production. Within the frame of pathogen-associated molecular pattern recognition by innate cells, cholinergic agents such as nicotine may alter innate receptor signaling pathways in these cells, emerging as an important issue in the sense of a reduced host capability to cope with pathogen infections. Despite our increasing knowledge about the extraneuronal cholinergic-immunological communication, several issues remain. For instance, it remains to be more clearly defined how these various communicative processes interact with each other; under what circumstances one of them may dominate or revert in relation to the others; and what conditions would be decisive for a shift in this type of interactions. Far from constituting an academic exercise, ACh and cholinergic agents are relevant immunomodulators that need to be better understood in the context of immunomodulation and merit future research. The data derived from upcoming research will surely impact on the therapeutic strategies for chronic inflammatory disease.

Conflict of interest

There are no financial conflicts of interests to declare in relation to this manuscript.

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