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Non-neuronal cholinergic system in regulation of immune function with a focus on α7 nAChRs

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ABSTRACT

In 1929, Dale and Dudley described the first reported natural occurrence of acetylcholine (ACh) in an animal's body. They identified this ACh in the spleens of horses and oxen, which we now know suggests possible involvement of ACh in the regulation of lymphocyte activity and immune function. However, the source and function of splenic ACh were left unexplored for several decades. Recent studies on the source of ACh in the blood revealed ACh synthesis catalyzed by choline acetyltransferase (ChAT) in $CD4^+$ T cells. T and B cells, macrophages and dendritic cells (DCs) all express all five muscarinic ACh receptor subtypes (mAChRs) and several subtypes of nicotinic AChRs (nAChRs), including α7 nAChRs. Stimulation of these mAChRs and nAChRs by their respective agonists causes functional and biochemical changes in the cells. Using AChR knockout mice, we found that M_1/M_5 mAChR signaling up-regulates IgG_1 and pro-inflammatory cytokine production, while α7 nAChR signaling has the opposite effect. These findings suggest that ACh synthesized by T cells acts in an autocrine/paracrine fashion at AChRs on various immune cells to modulate immune function. In addition, an endogenous allosteric and/or orthosteric α7 nAChR ligand, SLURP-1, facilitates functional development of T cells and increases ACh synthesis via up-regulation of ChAT mRNA expression. SLURP-1 is expressed in $CD205^+$ DCs residing in the tonsil in close proximity to T cells, macrophages and B cells. Collectively, these findings suggest that ACh released from T cells along with SLURP-1 regulates cytokine production by activating α7 nAChRs on various immune cells, thereby facilitating T cell development and/or differentiation, leading to immune modulation.

1. Brief history of acetylcholine identification in animal tissues

No one would argue that neurotransmitter acetylcholine (ACh) is not crucially important to the physiology of animals ranging from nematodes to humans. However, the history of ACh's discovery demonstrates that it is much more than just a neurotransmitter [1–4]. This review was undertaken to facilitate understanding of the important roles played by non-neuronal cholinergic systems in the regulation of immune function.

As early as 1867, Adolf von Baeyer chemically synthesized ACh, but it was then left for nearly four decades without exploration of its biological activity [5]. In 1906, Hunt and Taveau [6] discovered the extraordinary strong hypotensive action of ACh in rabbits during their study of the physiological actions of choline derivatives. This was after Mott and Halliburton (1899) [7] reported their identification of choline in the cerebrospinal fluid of patients with epilepsy and other nervous system diseases, and the fall of blood pressure elicited by choline in dogs, cats and rabbits. A little later, Henry Dale observed that some ergot extracts caused a decrease in blood pressure like that elicited by muscarine. He then asked a colleague, chemist Arthur J. Ewins, to purify the chemical entity that caused the hypotension, suspecting the ergot extracts had been contaminated with muscarine (see Burgen [8]). Ewins (1914) [9] succeeded in isolating a small amount of the hypotensive substance from ergot, fruiting bodies of *Claviceps purpurea*, an ergot fungus, and identified it physiologically and chemically as ACh. This was the first discovery of ACh in a natural organism. Dale (1914) [10] subsequently investigated the physiological effects of various choline esters, including ACh, and found that they possessed two distinct types of activity, muscarinic and nicotinic, and that ACh showed the greatest activity. Despite these findings, Dale was initially reluctant to suggest that ACh functioned as a neurotransmitter, because ACh had not yet been found as a normal constituent of the animal body (see Todman [11]).

It was Otto Loewi (1921) [12] who provided definitive physiological evidence for chemical transmission of vagus nerve impulses in a

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series of experiments using ingeniously designed frog heart preparations. He named the active substance "Vagusstoff," which was later pharmacologically identified by Loewi and Navratil (1926) [13] as ACh. Finally, in 1929, Dale and Dudley [14] successfully extracted and chemically identified ACh as a natural product present in the spleens of horses and oxen.

On the basis of the results summarized above and other observations, Dale came to the conclusion that there could be no doubt that liberation of ACh induced when a preganglionic or postganglionic parasympathetic impulses arrived at a synapse plays an essential part in the transmission of excitation to autonomic ganglion cells or at neuroeffector junctions. Dale [15,16] suggested the term "cholinergic" to describe the pre- and postganglionic autonomic neuronal fibers that transmitted their action through release of ACh, along with "adrenergic" for those that utilized a substance resembling adrenaline. In 1936, The Nobel Prize in Physiology or Medicine was awarded to Sir Henry Hallett Dale and Otto Loewi for their discoveries relating to the chemical transmission of nerve impulses. On the other hand, the splenic ACh discovered by Dale is of non-neuronal origin, as there is no evidence for the cholinergic innervation of the spleen [17–19]. It is therefore important to recognize that non-neuronal ACh was crucial for the establishment of the theory of chemical transmission of cholinergic nerve impulses.

2. Rationale for investigating cholinergic regulation of immune function

The presence of lymphocytes in the spleen and their circulation through the blood to the spleen were already known by the late 1910s [20,21]. Therefore, the identification of splenic ACh by Dale and Dudley [14] suggests the possibility that ACh is produced by lymphocytes (T and B cells), which are significant constituents of spleen cells, and that circulating peripheral blood contains a certain amount of ACh. In the early 1930s, both German [22,23] and British research groups [24,25] detected ACh in ox blood. However, large discrepancies among the groups' estimations of the ACh content of the blood led to suspension of the research, most likely due to the absence of a sensitive and specific method for determining ACh levels, as well as the lack of proper acetylcholinesterase (AChE) inhibitors. As a result, the source and function of splenic ACh were left unexplored for several decades.

Then in 1987, using a sensitive and specific radioimmunoassay (RIA) for ACh [26] and paraoxon, an instantaneously active AChE inhibitor, we detected 456 ± 53 pg/ml of ACh in the plasma of young women (n = 32) [27]. We then subsequently confirmed the presence of ACh in blood and plasma from various mammalian species, including humans (Table 1) [27–32]. The ACh levels in bovine blood were about 40 times higher than in human, and the levels in equine blood were about 10 times higher. Lower levels of ACh were also detected in the blood and plasma of other animal species.

Table 1
ACh content in blood and plasma of various mammalian species.

Species	ACh (pmol/ml)	
	Blood	Plasma
Cattle		
Holstein[a] (7)	360.25 ± 59.71	7.14 ± 2.55
Holstein[b] (5)	426.11 ± 18.38	5.55 ± 1.47
Japanese black[a] (8)	354.30 ± 18.14	15.08 ± 4.53
Chimpanzee (6)	21.50 ± 2.60	1.26 ± 0.07
Dog (10)	1.37 ± 0.23	0.13 ± 0.04
Goat (5)	4.05 ± 0.93	0.22 ± 0.06
Horse (5)	93.8 ± 16.3	0.66 ± 0.23
Human	8.66 ± 1.02 (30)	3.12 ± 0.36 (32)
Porcine (5)	11.70 ± 1.58	15.40 ± 1.58
Rat (10)	1.43 ± 0.20	0.75 ± 0.11
Rabbit (7)	24.50 ± 6.14	0.96 ± 0.11
Sheep (5)	2.04 ± 0.20	0.27 ± 0.05

Values are means ± SEM. Numbers of samples are in parentheses. Arranged from the data presented in References [27–32]. [a]Samples obtained from the ranch affiliated to the Animal Husbandry Experiment Station, Faculty of Agriculture, University of Tokyo. [b]Samples obtained from the ranch at Nisshin Flour Milling Co. Ltd.

3. Origin of blood ACh

In normal Japanese white rabbits, the ACh content of the blood cell fraction was about 25 times higher than in the plasma (3,722 vs. 140 pg/ml). Under partial inhibition of AChE with neostigmine (10 μg/kg, iv), nicotine (100 μg/kg, iv) caused an increase of the plasma ACh concentration that persisted for more than 15 min and was associated with a gradual reduction in the ACh content of the blood cell fraction [30]. This suggests nicotine induced ACh release from the blood cells into the plasma.

In another experiment, the plasma ACh content of rabbits immunized with an ACh-immunogen was about 80 times higher than that in untreated rabbits (11,359 vs. 140 pg/ml of blood, n = 8 and 7, respectively), whereas no difference was observed between the ACh contents of the blood cell fractions from the two groups (4,146 vs. 3,722 pg/ml of blood, n = 8 and 7, respectively) [30]. This also suggests plasma ACh originates from blood cells, and that a large portion of plasma ACh found in the immunized rabbits is bound to the antibody and thus protected from hydrolysis by AChE. Moreover, using the procedure of Fonnum [33], the presence of ACh synthesizing activity was detected in rabbit buffy coat layer cells [34], human mononuclear leukocytes (MNLs) [35], rat lymphocytes and human leukemic cell lines [36], further supporting the idea that ACh detected in plasma originates from blood cells.

On the basis of the above findings, we endeavored to identify which type(s) of blood cells contains ACh by measuring the ACh content of human MNLs, polymorphonuclear leukocytes (PMNs) and red blood cells [31]. We found that about 60% of the ACh content in whole blood is present within MNLs (1263.5 ± 149.0 vs. 731.0 ± 85.8 pg/ml blood, mean ± SEM, n = 30), and that there is a significant correlation between the ACh content of whole blood and that of MNLs (r = 0.91; $P < 0.001$). No ACh was detected in PMNs or red blood cells. These results indicate that the major portion of blood ACh originates from circulating MNLs constituted mainly of lymphocytes and a small monocyte fraction.

3.1. ACh synthesis in T cells

Tucek [37] reported that in peripheral tissues of the rat, ACh is synthesized by both choline acetyltransferase (ChAT, EC 2.3.1.6) and carnitine acetyltransferase (CarAT, EC 2.3.1.7). This means that ACh synthesis in peripheral tissues determined using the method of Fonnum [33] reflects the sum of ChAT and CarAT activities.

3.1.1. ChAT-catalyzed ACh synthesis in T cells

To identify which types of lymphocytes or monocytes synthesizes and releases ACh, we evaluated the ACh content, ChAT and CarAT activities, and expression of ChAT mRNA using human leukemic cell lines as models (Table 2) [38–40]. Different amounts of ACh were detected in all cell lines tested, except BALL-1 cells, a B cell line, and both ChAT and CarAT activities were expressed (Table 2). However, expression of ChAT mRNA was detected only in T cell lines, and the ACh content correlated well with ChAT activity (r = 0.960, n = 13), but not with CarAT activity (r = −0.059, n = 5), suggesting ChAT is responsible for ACh synthesis in lymphocytes.

Using bromoACh (BrACh), a ChAT inhibitor, and bromoacetylcarnitine (BrACar), a CarAT inhibitor, with MOLT-3, HSB-2 and CEM T cells, we examined whether ChAT or CarAT mediates the up-regulation of ACh synthesis induced by phytohemagglutinin (PHA) stimulation [38]. We found that 10 μM BrACh reduced ACh synthesis by about 30–50% and that 100 μM BrACh reduced it by about 50–70%. On the other hand, 100 μM BrACar inhibited ACh synthesis by a maximum of about 30%.

Table 2
ACh content, ChAT and CarAT activities, and ChAT mRNA expression in human leukemic cell lines.

Cell Line	Cell Type	ACh Content pmol/10^6 cells	ChAT Activity pmol/mg protein/min	CarAT Activity pmol/mg protein/min	ChAT mRNA expression
CCRF-CEM	T	12.6 ± 0.6 (4)	2.9 ± 0.2 (7)	22.8 ± 4.6 (7)	+
HPB-ALL	T	0.7 ± 0.1 (13)	0.7 ± 0.1 (3)	NT	+
HSB-2	T	36.2 ± 3.5 (7)	1.4 ± 0.1 (11)	58.3 ± 15.3 (3)	+
HUT-78	T	0.4 ± 0.1 (7)	2.5 ± 0.4 (8)	NT	+
Jurkat	T	8.2 ± 0.4 (4)	4.3 ± 0.8 (3)	17.2 ± 1.9 (6)	+
MOLT-3	T	251.5 ± 34.9 (7)	22.4 ± 3.0 (4)	53.3 ± 5.6 (3)	+
MOLT-4	T	38.8 ± 5.9 (8)	8.0 ± 1.0 (5)	NT	+
BALL-1	B	ND (4)	0.4 ± 0.2 (3)	NT	-
Daudi	B	1.2 ± 0.1 (4)	0.1 ± 0.02 (4)	125.6 ± 44.2 (3)	-
NALM-6	B	0.04 ± 0.01 (9)	0.1 ± 0.02 (4)	NT	-
NALM-16	prelymphoma	0.3 ± 0.1 (4)	0.1 ± 0.06 (8)	NT	-
REH	prelymphoma	0.8 ± 0.01 (3)	0.2 ± 0.03 (6)	NT	-
U937	monocytic	0.02 ± 0.01 (4)	0.2 ± 0.03 (8)	NT	-

Values are means ± SEM. Number of experiments is shown in parenthesis. ND, not detectable. NT, not tested. +, positive. −, negative. Rearranged from the data presented in References [38–40].

These results demonstrate that immunological stimulation of ACh synthesis in T cells is dependent on ChAT. They also show that it is important to differentiate whether ACh synthesis in the target cells is catalyzed by ChAT or CarAT, and whether it is related to the functional activity of the cells.

3.1.2. ChAT mRNA expression in T cells

As mentioned, Fujii et al [41] provided the first definitive evidence for ChAT-catalyzed ACh synthesis in T cells by demonstrating the expression of ChAT mRNA and protein in the MOLT-3 human leukemic T cell line. Later, the expression of ChAT mRNA and ACh synthesis were confirmed in other human leukemic T cell lines, as well as in human MNLs [39,40] and rat lymphocytes [42].

3.1.3. Mechanisms controlling ACh synthesis in T cells

PHA, which activates T cells via the T cell receptor (TCR)/CD3 complex, increases ACh synthesis by up-regulating ChAT mRNA expression [39,40]. This finding suggests immunological stimulation via TCR/CD3, such as occurs upon interaction of T cells with antigen-presenting cells, activates lymphocytic cholinergic activity. In addition to PHA, phorbol 12-myristate 13-acetate, a protein kinase C activator; A23187 or ionomycin, Ca^{2+} ionophores; and dibutyryl cAMP (dbcAMP) all up-regulate ChAT mRNA expression and increase ACh synthesis in human leukemic T cell lines and human MNLs [43,44]. PHA-induced ChAT mRNA expression is inhibited by FK506, an immunosuppressant calcineurin inhibitor, which suggests the involvement of calcineurin-mediated pathways in the regulation of ChAT transcription [44]. Furthermore, PHA also up-regulates gene expression for M_5 muscarinic ACh receptor (mAChR) and AChE [45]. These findings lend further support to the idea that T cell-derived ACh is involved in the regulation of immune function.

3.2. Mechanism of ACh release from T cells

Although several theories have been proposed, the mechanisms involved in non-neuronal ACh storage and release remain unclear. Exocytosis is suggested by the identification of vesicular acetylcholine transporter (VAChT) expression in monkey bronchial epithelial cells and human small cell lung carcinoma cells. On the other hand, active transport by organic cation transporters has been detected in human placenta, mouse and human urothelium and mouse bronchial epithelium (see a review by Wessler and Kirkpatrick [4]).

It is not known whether ACh in T cells is stored in vesicles. Fujii et al [39] did not detect VAChT gene expression in human MNLs, which argues against a VAChT-based mechanism. An alternative is that ACh is synthesized in T cells when necessary and then directly released without storage. Consistent with that idea, Fujii et al [46] detected the mRNA and protein expression of mediatophore in T cells. Mediatophore is a homo-oligomer of a 16-kDa subunit homologous with the proteolipid subunit c of vacuolar H^+-ATPase (V-ATPase), which is involved in ACh release at the Torpedo nerve-electroplaque junction [47]. Moreover, immunological activation of T cells by PHA up-regulated mediatophore gene expression, while transfection of a small interference RNA (siRNA) targeting mediatophore knocked down its expression and reduced ACh release. ACh release from T cells thus appears to be mediated, at least in part, by mediatophore.

4. Expression of AChRs in immune cells

Most immune cells express subtypes of both mAChRs and nicotinic ACh receptors (nAChRs), suggesting ACh synthesized and released from T cells acts on these AChRs via autocrine/paracrine pathways and is involved in regulating immune function (see reviews [17,48–50]). Earlier reviews on AChRs in lymphocytes have been published by Homo-Delarche and Durant [51], Maslinski [52], Nordberg [53] and Plaut [54]. At the time of their publication, however, neuronally derived ACh was postulated to activate AChRs on immune cells, despite the fact that ACh is hydrolyzed by AChE within a few milliseconds after its release from nerve endings [50].

All five mAChR subtypes (M_1-M_5) are expressed on T and B cells, dendritic cells (DCs) and macrophages [55,56]. Because of a wide variety of nAChR subtypes, details of nAChR subtype expression in each immune cell type are not yet known. However, most immune cells express mRNAs for neuronal type nAChR subunits, though the patterns of nAChR subunit expression vary among animal species and immune cells, and even between developmental stages within a cell [57]. Gene expression for the α3, α5, α7, α9 and α10 nAChR subunits has been commonly detected in human T and B cells [49]. However, data on nAChR subunit expression detected using immunohistochemical techniques should be interpreted with caution, as most commonly available antibodies against α3, α4, α7, β2 and β4 subunits sometimes generate false positive results [58].

4.1. AChRs and the regulation of immune function

Early studies of the functions of AChRs in immune cells revealed that stimulation of mAChRs and nAChRs using their respective agonists elicited a variety of functional and biochemical effects, including enhanced cytotoxicity and increased cGMP formation and intracellular Ca^{2+} signaling, which suggests AChRs are involved in regulating immune function (see a review [48]).

4.1.1. mAChRs

Stimulation of human leukemic T cell (CCRF-CEM) and B cell (Daudi) lines using ACh or the mAChR agonists bethanechol and oxotremorine-M induced rapid Ca^{2+} transient followed by extracellular Ca^{2+}-dependent oscillations and up-regulated c-fos gene expression [59]. These findings are consistent with the notion that ACh released from T cells acts on T and B cell mAChRs via autocrine/paracrine pathways to trigger nuclear signaling, leading to modulation of immune function.

$CD8^+$ T cells from M_1 mAChR-deficient (M_1 mAChR-KO) mice showed a defect in their ability to differentiate into cytolytic T lymphocytes [60]. By contrast, no defect was observed when expansion of $CD8^+$ T cells from M_1 or M_5 mAChR-KO mice was stimulated by infection with lymphocytic choriomeningitis virus or vesicular stomatitis virus [61]. Apparently M_1 and M_5 mAChRs play no role in antiviral immunity.

On the other hand, Fujii et al [62] detected less antigen-specific IgG_1 production in M_1/M_5 mAChR-KO mice immunized with ovalbumin than was seen in similarly immunized wild-type mice. Furthermore, upon stimulation with the antigen, splenic cells from M_1/M_5 mAChR-KO mice produced less interferon-γ and interleukin (IL)-6, two pro-inflammatory cytokines, than did splenic cells from wild-type mice. These findings suggest M_1/M_5 mAChRs are involved in up-regulating the immune response to protein antigens by increasing production of pro-inflammatory cytokines.

4.1.2. nAChRs

Nicotine stimulation of human leukemic T and B cell lines expressing various nAChR subunits [55] elicits a transient Ca^{2+} signal that is effectively antagonized by α-bungarotoxin (α-BTX) or methyllycaconitine (MLA), two selective α7 nAChR antagonists, which indicates α7 nAChRs are at least partly responsible for the observed Ca^{2+}-signaling [63]. Razani-Boroujerdi et al [64] reported that α7 nAChRs on T cells require functional T cell receptor (TCR)/CD3 and leukocyte-specific tyrosine kinase to mediate nicotine-induced Ca^{2+}-signaling via Ca^{2+} release from intracellular stores, which is insensitive to α7 nAChR antagonists such as α-BTX and MLA. However, a recent study by Fujii et al [65] showed that MLA reversed the growth attenuation elicited by PHA and abolished the PHA-induced increase of ACh synthesis in MOLT-3 cells. Given that PHA activates T cells via the TCR/CD3 complex and increases ACh synthesis through up-regulation of ChAT mRNA expression via calcineurin-mediated pathways [39,40], the results suggest that α7 nAChRs linked to TCR/CD3 are sensitive to MLA. The reason for the apparent differences in α7 nAChR sensitivity to MLA in T cells remains unclear.

It is well documented that α7 nAChRs mediate down-regulation of TNF-α synthesis in macrophages, thereby suppressing inflammatory responses [66,67]. Furthermore, Fujii et al [68] showed that production of antigen-specific IgG_1 antibody and the pro-inflammatory cytokines TNF-α, IFN-γ and IL-6 is elevated in α7 nAChR-KO mice. This suggests the involvement of α7 nAChRs in the regulation of immune responses through modulation of cytokine synthesis.

5. SLURP-1 and the regulation of immune function

Endogenously expressed secreted lymphocyte antigen-6/urokinase-type plasminogen activator receptor-related peptide (SLURP)-1 was first purified from human blood and identified by Adermann et al [69]. SLURP-1 acts as a positive allosteric ligand, potentiating the action of ACh at α7 nAChRs in *Xenopus* oocytes expressing recombinant human α7 nAChRs [70], or as an orthosteric agonist for α7 nAChRs, stimulating pro-apoptotic activity and differentiation in human keratinocytes [71]. SLURP-1 gene mutations have been detected in patients with Mal de Meleda (MdM), a rare autosomal recessive skin disorder characterized by transgressive palmoplantar keratoderma [72].

SLURP-1 mRNA is expressed in most immune organs, including the thymus and spleen [73], as well as in peripheral blood MNLs, DCs and macrophages, suggesting a role for SLURP-1 in the regulation of immune function [56,73]. Indeed, evidence from a study using T cells from MdM patients with a genetic SLURP-1 mutation [74] suggest SLURP-1 plays a crucial role in the T cell activation.

Fujii et al [65] detected SLURP-1 expression in $CD205^+$ DCs (Fig. 1A, B) residing mainly in the interfollicular zone surrounding the germinal center of human tonsils. Furthermore, tonsillar SLURP-1^+CD205^+ DCs are surrounded by $CD4^+$ T cells and other immune cells expressing markers for monocyte/macrophages (CD68) [75], DCs (CD11c) [76] and B cells (HLA-DOβ) [77] (Fig. 1C–F), which suggests close interaction between SLURP-1^+ DCs and $CD4^+$ T cells, certain DC subtypes, macrophages and B cells during antigen presentation and other immunological reactions. A recent study by Fukuya et al [78] on the critical role of $CD205^+$ DCs in the regulation of T cell immunity suggests SLURP-1 and ACh released from T cells act via α7 nAChRs to regulate immune function.

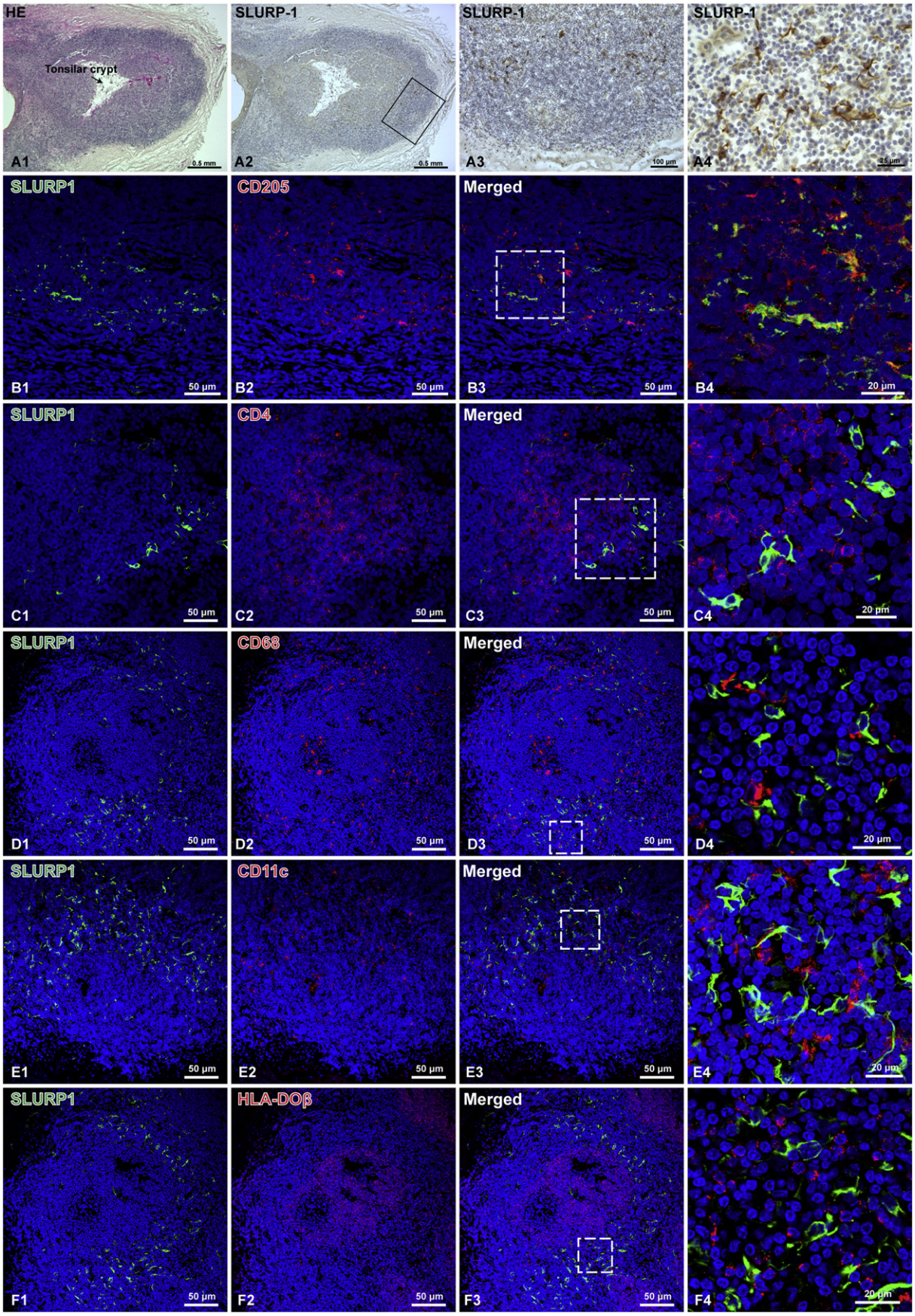
Recombinant SLURP-1 (0.5 μg/ml) attenuates proliferation of MOLT-3 cells and human blood MNLs, and increases the ACh content of the cells by up-regulating ChAT expression, all of which are abolished by MLA (Fig. 2) [65]. This suggests SLURP-1 activates cholinergic transmission by potentiating ACh synthesis and its action at α7 nAChRs, thereby facilitating the functional development of T cells. Chernyavsky et al [79] recently reported that SLURP-1 exerts anti-inflammatory effects by down-regulating TNF-α production in T cells and IL-1β and IL-6 secretion from macrophages. Taken together, these findings suggest activation of α7 nAChRs in T cells up-regulates ACh synthesis, which facilitates further action by ACh in the presence of SLURP-1 at α7 nAChRs expressed on DCs and macrophages, leading to modification of cytokine synthesis.

6. α7 nAChRs and the regulation of immune function

The schematic diagram in the Supplementary data (Fig. 3) illustrates the findings obtained so far on the non-neuronal cholinergic system and its potential role in T cell development and/or differentiation. $CD4^+$ T cells express most cholinergic components, including ChAT, mAChRs and nAChRs, including α7 nAChRs, which is sufficient to constitute an independent cholinergic system within the immune system [49,50]. DCs, macrophages, B cells also express both mAChRs and nAChRs, including α7 nAChRs, and $CD205^+$ DCs express SLURP-1 (Fig. 2) [56,65].

As described, antigen presentation between naïve $CD4^+$ T cells and SLURP-1^+ DCs activates T cells, leading to increased ACh synthesis as the result of up-regulated ChAT expression (Fig. 3). ACh released from T cells then acts in an autocrine/paracrine fashion at α7 nAChRs to modulate cytokine synthesis in T cells, DCs, macrophages and B cells. ACh

Fig. 1. Immunohistochemical staining of SLURP-1, CD205, CD4, CD68, CD11c and HLA-DOβ in human tonsil. A. SLURP-1 immunoreactivity. A1: Low-magnification images of a palatine tonsil (hematoxylin and eosin staining). A2: SLURP-1 immunohistochemistry with avidin-biotin-peroxidase staining. A3: Enlarged image of the boxed area in A2. SLURP-1^+ cells are seen mainly in the interfollicular zone surrounding the germinal center of the tonsil. A4: Higher power image of SLURP-1 labeling. SLURP-1^+ cells have a DC-like structure. B. SLURP-1 and CD205 immunoreactivities in the tonsil. B1: SLURP-1 immunoreactivity in the interfollicular zone. B2: CD205 immunoreactivity. B3: Merged image of the SLURP-1 and CD205 immunoreactivities. Some $CD205^+$ cells are also SLURP-1^+. B4: Enlarged image of the boxed area in B3. C. SLURP-1 and CD4 immunoreactivities in the tonsil. C1: SLURP-1 immunoreactivity in the interfollicular zone. C2: CD4 immunoreactivity. C3: Merged image of the SLURP-1 and CD4 immunoreactivities. The SLURP-1^+ cells are surrounded by $CD4^+$ T cells. C4: Enlarged image of the boxed area in C3. D. SLURP-1 and CD68 (monocyte/macrophage) immunoreactivities in the tonsil. D1: SLURP-1 immunoreactivity in the interfollicular zone. D2: CD68 immunoreactivity. D3: Merged image of the SLURP-1 and CD68 immunoreactivities. D4: Enlarged image of the boxed area in D3. The SLURP-1^+ cells are localized in close proximity to the $CD68^+$ cells. E. SLURP-1 and CD11c (certain DC subtypes) immunoreactivities in the tonsil. E1: SLURP-1 immunoreactivity in the interfollicular zone. E2: CD11c immunoreactivity. E3: Merged image of the SLURP-1 and CD11c immunoreactivities. E4: Enlarged image of the boxed area in E3. The SLURP-1^+ cells are in close proximity to the $CD11c^+$ cells. F. SLURP-1 and HLA-DOβ (B cells) immunoreactivities in the tonsil. F1: SLURP-1 immunoreactivity in the interfollicular zone. F2: HLA-DOβ immunoreactivity. F3: Merged image of the SLURP-1 and HLA-DOβ immunoreactivities. F4: Enlarged image of the boxed area in F3. The SLURP-1^+ cells are in close proximity to the HLA-$DO\beta^+$ cells. Adopted from Fujii et al [65] with modifications.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Tonsilar crypt
A1
0.5 mm
SLURP-1
A2
0.5 mm
SLURP-1
A3
100 μm
SLURP-1
A4
25 μm
SLURP1
CD205
Merged
B1
50 μm
B2
50 μm
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CD4
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SLURP1
CD68
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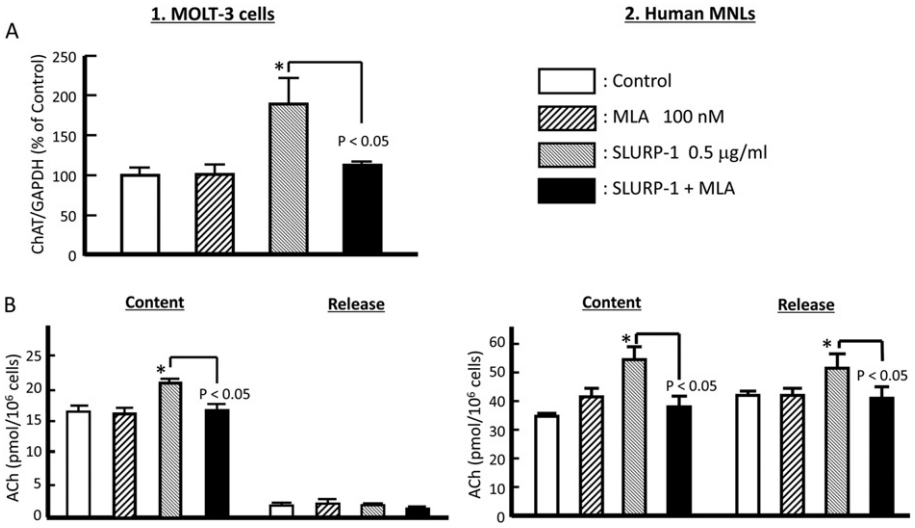

Fig. 2. Effects of SLURP-1 and methyllycaconitine (MLA), a specific α7 nAChR antagonist, on (A) ChAT gene expression and (B) intracellular ACh content and release in MOLT-3 cells and human MNLs. Cells were incubated for 48 h with SLURP-1 (0.5 μg/ml) and/or MLA (100 nM). A: ChAT mRNA expression analyzed using RT-PCR. The expected size of the ChAT RT-PCR product was 652 bp. The bars represent means ± SEM (n = 3) of the levels of ChAT mRNA normalized to the level of GAPDH mRNA and expressed as a percentage of Control. *P < 0.05 vs. Control (Student's t-test). B: ACh was measured within the cells and in conditioned medium using a specific RIA [26] (Kawashima et al, 1980). All assays were carried out in triplicate. Data are means ± SEM (n = 5). *P < 0.05 vs. Control (Tukey's t-test). Adopted from Fujii et al [65] with modification.

acting along with SLURP-1 at α7 nAChRs on T cells stimulates further synthesis of ACh, as shown in Fig. 2. Then via α7 nAChRs on macrophages, ACh down-regulates synthesis of pro-inflammatory cytokines such as TNF-α [19,66,67,80]. SLURP-1 released from $CD205^+$SLURP-1^+ DCs should potentiate the action of ACh at α7 nAChRs on macrophages, enhancing its modulation of pro-inflammatory cytokine production. Oloffson et al [80] suggested α7 nAChRs expressed in non-T cell bone marrow-derived immune cells, together with splenic ACh-producing T cells, are necessary for normal function of the inflammatory reflex leading to modulation of TNF-α synthesis. As described above, however, α7 nAChRs on T cells also act within the inflammatory reflex by stimulating ACh synthesis and release, thereby regulating pro-inflammatory cytokine production by macrophages [65].

Chernyavsky et al [81] showed that SLURP-1 up-regulates NF-κB expression in Het-1A human oral keratinocytes via α7 nAChR-mediated pathways, which involve both ionic events and activation of protein kinases. It is therefore reasonable to consider that ACh/SLURP-1-induced activation of α7 nAChRs on immune cells is also mediated via two signaling systems entailing both ionic events and protein kinase cascades.

Moriwaki et al (in preparation) discovered that IL-22 up-regulates expression of SLURP-1 mRNA in human keratinocytes, and that this effect is suppressed by S3I-201, an inhibitor of the transcription factor STAT3, as well as by siRNA targeting STAT3. $CD4^+$ T cells, which produce IL-17, also have the ability to synthesize IL-22 [82]. Thus, IL-22 released from T cells should stimulate SLURP-1 production, leading to further activation of α7 nAChRs on immune cells residing in the vicinity of $CD205^+$SLURP-1^+ DCs.

The spleen, lymph nodes and tonsils are secondary lymphoid organs and have many functional and structural similarities, such as lymphoid follicles, a germinal center and a marginal zone containing T and B cells, DCs and macrophages. It would therefore be reasonable to extrapolate the findings made in tonsils to the spleen and lymph nodes. Furthermore, tonsils were recently shown to play a key role in T cell development [83].

Taken together, the accumulated evidence suggests that α7 nAChRs on immune cells play key roles in the regulation of immune function by modulating the synthesis of various cytokines as well as ACh, thereby regulating the development and differentiation of naïve CD4 T cells into Th1/Th2/Th17 and regulatory T cells.

At present, little is known about the effects exerted by ACh via α7 nAChRs on DCs and B cells, other than induction of Ca^{2+} signaling in B cells [63]. However, it is known that antigen-specific IgG_1 production is up-regulated in α7 nAChR-KO mice [68] and that α7 nAChRs and other nAChR subunits are involved in the regulation of B cell development and activation [84].

As described above, the role of α7 nAChRs in the regulation of pro-inflammatory cytokine production and immune function has been investigated primarily in studies using α7 nAChR-KO mice [66,68]. However, because human T and B cells express mRNAs encoding both α7 and α9 nAChR subunits [49], and because both α-BTX and MLA exhibit high affinity not only for α7 nAChRs but also for α9 nAChRs [85,86], we cannot rule out the possibility that the effects of ACh, nicotine and SLURP-1 that are blocked by α7 nAChR antagonists such as α-BTX and MLA may also be mediated in part by α9 nAChRs.

7. Conclusion

Viewed in the context of what we now know, it is apparent that the splenic ACh identified by Dale and Dudley (1929), the first ACh discovered naturally occurring in an animal's body, was likely involved in regulating immune function. Several decades later, we discovered ChAT-catalyzed ACh synthesis in T cells, a significant constituent of spleen cells, and the up-regulation of ChAT gene expression through immunological activation of T cells. Immune cells express all five mAChR subtypes and several nAChR subtypes, including α7 nAChRs. Stimulation of these mAChRs and nAChRs by their respective agonists elicits functional and biochemical changes in immune cells, suggesting the involvement of ACh synthesized by T cells in the regulation of immune function. Furthermore, studies of AChR-KO mice showed that M_1/M_5 mAChRs mediate up-regulation of antigen-specific IgG_1 and pro-inflammatory cytokine production, while α7 nAChRs mediate the opposite effect. In addition, a recently discovered endogenous α7 nAChR allosteric and/or orthosteric ligand, SLURP-1, facilitates functional development of T cells via α7 nAChR-mediated pathways, leading to increases in ACh synthesis. Collectively, these findings suggest that, acting via α7 AChRs, ACh released from T cells contributes to the regulation cytokine production in immune cells, thereby modulating T cell development and/or differentiation.

Conflict of Interest

The authors declare that they have no conflict of interest.

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