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ORIGINAL RESEARCH

Functional role of the nicotinic arm of the acetylcholine regulatory axis in human B-cell lines

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Abstract: We studied the involvement of nicotinic acetylcholine receptors (nAChRs) in the inflammation-related activity of human B-cell lines. Activation of nAChRs in Daudi cells with epibatidine abolished the pansorbin-dependent upregulation of the pro-inflammatory marker Cox-2 both at the mRNA and protein levels, indicating that the nicotinergic signaling suppresses B-cell activation. While the anti-inflammatory action on B-cells was mediated predominantly through α7 nAChR, as could be judged from abolishing epibatidine effects with methyllycaconitine, both α7 and non-α7 nAChRs, such as α2-containing receptors, were involved in regulation of B-cell apoptosis. The net effect was antiapoptotic. To determine the role of nAChRs in regulating B-cell activation/plasmacytic differentiation, we measured changes in the CD38, CD138 and Bcl-6 gene expression. Epibatidine significantly ($P < 0.05$) upregulated CD38 at the transcriptional level and CD138 and Bcl-6 – at the translational levels. AR-R17779 significantly ($P < 0.05$) increased the protein levels of CD38 and CD138. In both cases, the effect of epibatidine was abolished with Mec, and that of AR-R17779 – by MLA, demonstrating a functional role of nAChRs in regulating Daudi cell differentiation. The obtained results revealed distinct contributions of α7 and non-α7 nAChRs to regulation of B-cell activation/differentiation, and suggested that signaling through the nicotinic arm of acetylcholine regulatory axis is important for B-cell involvement in inflammation.

Keywords: Daudi B cell line, nicotinic acetylcholine receptor, inflammation, apoptosis, pansorbin

Introduction

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated cation channels initially discovered in nerve and muscle cells and now being intensively studied in non-excitable cells of various origin.[1] Structurally, nAChRs expressed in neuronal and non-neuronal cells are pentamers composed of different combinations of nine alpha (α2–α10) and three beta (β2–β4) subunits, with each subunit combination possessing certain pharmacologic sensitivity and regulating specific cell functions.[2] The nAChRs expressed by immune cells can be activated by acetylcholine either released from innervating nerve terminals or endogenously produced and secreted by nonneuronal cells.[3] Acetylcholine was reported to be proinflammatory for lymphocytes and epithelial cells, anti-inflammatory for mast cells and macrophages, both pro- and anti-inflammatory for monocytes, and variable for neutrophils and eosinophils.[4] Stimulation of α7 nAChR with vagus-produced acetylcholine can attenuate the proinflammatory cytokine production.[5,6] This receptor may mediate the anti-inflammatory effects of nicotine and other nAChR agonists.[7] However, the

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mechanisms by which nAChRs control inflammation and the cells involved in this process remain to be elucidated.

B lymphocytes are unique cells producing antibodies in response to antigenic stimuli. In addition, B-cells are considered the important players in regulating inflammation, because they can produce both proinflammatory (interleukin-6 [IL-6], tumor necrosis factor alpha [TNFα], IL-12) and anti-inflammatory (IL-10) cytokines.[8] Both normal B lymphocytes and some B lymphocyte-derived cell lines express various types of acetylcholine receptors.[3] The nAChRs have been shown to regulate the development and survival of B lymphocytes.[9,10] Nicotine stimulated proliferation of B lymphocyte-derived cell lines and normal plasma cells and decreased antibody production.[11,12] Recent findings suggested an important role for α7 nAChR in the regulation of B-cell functions. Skok and colleagues[13] found reduced preimmune level of serum immunoglobulin G (IgG) in α7 knockout mice, whereas Fujii and colleagues[14] demonstrated that the α7 nAChR expressed in murine immune cells exhibits negative regulation of antibody synthesis and controls the regulatory cytokine production. Stimulation of human leukemic B-cell lines expressing the α2, α5, α6, α7, α9, α10, and β2 and β4 nAChR subunits with nicotine or epibatidine elicited a transient Ca^{2+} signaling that was antagonized effectively by α-bungarotoxin, suggesting that the α7 nAChR is, at least partly, responsible for nicotine-induced Ca^{2+} signaling in lymphocytes.[3,15]

The purpose of this study was to determine the involvement of α7 *vs* non-α7 nAChRs in regulation of the inflammation- and apoptosis-related events in human B cells.

Materials and methods
Cells and reagents
The Daudi cells – a human Burkitt's lymphoma cell line – was purchased from ATCC (Catalog # CCL-213) and grown in the ATCC complete growth medium (Catalog #30-2001) at 37 °C in a humid, 5% CO_2 incubator. Another Burkitt's lymphoma cell line, Ramos, as well as the line, REH, corresponding to human pre-B lymphocytes were a kind gift of Dr Sidorenko (Kavetzky Institute of Experimental Oncology, Kiev, Ukraine). The cells were grown in RPMI-1640 medium supplemented with 20 mM HEPES, 40 µg/ml gentamicin, and 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO). The nicotinic ligands epibatidine, mecamylamine (Mec), methyllycaconitine (MLA) as well as 3-(4,5-dimethylthriasol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were also from Sigma-Aldrich.

AR-R17779 was a gift from AstraZeneca Pharmaceuticals (Wilmington, DE). Pansorbin was purchased from EMD Chemicals, Inc. (Cat. No. 507862; Gibbstown, NJ). Fluorescein isothiocyanate (FITC)-labeled anti-CD19 (Cat. No. IM 1284) and phycoerythrin (PE)-labeled anti-CD22 antibodies (Cat. No. IM1835) were from Immunotech (Marseille, France). PE-labeled Streptavidin (Cat. No 554061) was from BD Biosciences (Bioline LCC, Kiev, Ukraine).

Flow cytometry experiments
The Daudi, Ramos, and REH cells were suspended to a concentration of 1×10^6 cells per tube in 50 µl of 1% bovine serum albumin (BSA)-containing phosphate-buffered saline (PBS), and treated with biotinylated rabbit nAChR antibodies for 15 min at room temperature. The antibodies used in this study were raised against short synthetic fragments of the rat nAChR subunits α4, α7, β2, or β4 and shown to bind corresponding nAChR subunits in rat and guinea-pig autonomic ganglia[16,17] and mouse B lymphocytes.[10,11] The sequences used to generate the α4, β2, and β4 antibodies are identical between human and rat. Since the peptide used to raise anti-α4 antibody, AVGTYNTRKYEC, is highly homologous to the corresponding fragment of rat and human α2 subunit (ATGTYNSKKYDC; www.uniprot.org), this antibody also reacts with α2 nAChR. The α7 antibody also recognizes human receptor because the peptide used for immunization contained only two conservative replacements: Lys for Arg and Ser for Asn. The specificity of α7 antibody was confirmed in experiments with α7 knockout mice (data not shown). The antibody concentration was selected according to ELISA with corresponding antigenic peptides, as detailed elsewhere.[16,17] After a thorough wash with PBS, the cells were stained with PE-labeled streptavidin for 15 min, washed by centrifugation and analyzed on the EPICS-XL flow cytometer (Coulter-Beckman, Fullerton, CA) using appropriate software. The cell aliquots stained with FITC-labeled anti-CD19 and PE-labeled anti-CD22 were analyzed similarly in a flow cytometer.

Proliferation studies
The Daudi, Ramos, and REH cells seeded in 96-well plates, 1.5×10^4 cells per well in 100 µl of culture medium, were incubated with epibatidine at 37 °C and 5% CO_2 for 48 hours. The number of live cells was measured by MTT inclusion according to Carmichael and colleagues.[18] Briefly, MTT was added to the culture medium to the final concentration of 0.4 mg/ml. After 4 hrs of incubation at 37 °C, the medium was removed, and the formazan crystals formed were diluted in

100 μl of dimethyl sulfoxide. After that, 25 μl of 0.1 M glycine, 0.1 M NaCl, pH 10,5 were added to each well, and the absorbance at 545 nm was measured using StatFax 2100 microplate reader (Awareness Technology, Los Angeles, CA).

Real-time quantitative polymerase chain reaction (qPCR) experiments

Total RNA was extracted from Daudi cells at the end of exposure experiments with the RNeasy Mini Kit (Qiagen, Valencia, CA) and used in the qPCR assay detailed elsewhere.[19] All qPCR primers were designed with assistance from Primer Express software (version 2.0; Applied Biosystems, Foster City, CA) and the Assays-on-Demand service provided by Applied Biosystems. The qPCR reactions were performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems) and the TaqMan Universal Master Mix reagent (Applied Biosystems) in accordance to the manufacturer's protocol, as described by us in detail elsewhere.[20] To correct for minor variations in mRNA extraction and reverse transcription, the gene expression values were normalized using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The data from triplicate samples were analyzed with a sequence detector software (Applied Biosystems), and results expressed as fold of control determined in intact (control) cells, and taken as 1.

In-cell Western (ICW) assay

The ICW assay was performed as described by us in detail elsewhere[20] using reagents and equipment from LI-COR Biotechnology (Lincoln, NE). After incubation of 3×10^6 Daudi cell/well in a 96-well plate in culture media with or without test agents for 16 hours, the experimental and control Daudi cells were fixed *in situ*, washed, permeabilized with Triton solution, incubated with the LI-COR Odyssey Blocking Buffer for 1.5 hours and then treated overnight at 4 °C with a primary rabbit antibody to human cyclooxygenase-2 (Cox-2), Bcl-2, Bcl-6, Bax, CD38 or CD138 (all from Santa Cruz Biotechnology, Inc. Santa Cruz, CA). After that, the cells were washed and stained for one hour at room temperature with a secondary LI-COR IRDye 800CW goat anti-rabbit or IRDye 800CW anti-mouse antibodies, each diluted 1:800. Sapphire700 (1:1000) was used to normalize for cell number/well. The protein expression was then quantitated using the LI-COR Odyssey Imaging System.

Statistical analysis

All experiments were performed in duplicates or triplicates, and the results were expressed as mean ± standard deviation. Statistical significance was determined using Student's *t*-test. Differences were deemed significant if the calculated *P* value was <0.05.

Results
The level of expression of nAChRs depends on B lymphocyte differentiation

To select the most appropriate model for functional studies of B cell nAChRs, we evaluated nAChR expression in three human B cell lines representing different stages of B lymphocyte differentiation, ie, pre-B (REH), and mature B lymphocytes (Ramos) and B lymphoblasts (Daudi), using previously characterized rabbit affinity purified antibodies against the α2/α4, α7, β2, and β4 subunits.[16,17] The highest amount of antibody binding was observed in Daudi cells and the lowest in REH cells (Figure 1A). Because Daudi cells express α2, but not α4, subunit,[3] the reactivity of α2/α4 antibody was attributed to α2 nAChR. Since Daudi cells are much larger than REH cells, and since each assay used the same number of cells, the higher fluorescence values could be due to a larger cell size. However, Ramos cells, which are similar in size to REH cells, also expressed significantly more nAChRs than REH cells both at the protein (Figure 1A) and mRNA (Figure 1B) levels, suggesting that an increased nAChR expression was associated with B-cell maturation rather than differences in cell size. Indeed, we found that Ramos cells expressed significantly more B lymphocyte differentiation markers CD19 and CD22[21] than REH cells (Figure 1C). Therefore, the B cell lines corresponding to mature human B lymphocytes expressed more nAChRs on their plasma membrane than pre-B-cells. However, the two cell lines, Daudi and Ramos, both related to a similar type of malignancy (Burkitt's lymphoma) expressed different relative amounts of distinct nAChR subtypes. While in Daudi cells the ratio of the signals produced by α2/α4 *vs* α7 antibodies was 2.8, that in Ramos cells was 9.4, indicating that the former cell type possesses a relatively higher level of α7 nAChRs. Since α7 had been reported to play a major role in mediating acetylcholine effects on inflammation,[6,14,22,23] we selected Daudi cells for the functional studies described below.

Nicotinic signaling modulates the effects of pansorbin on the inflammatory and apoptosis markers in Daudi cells

To elucidate the role of nAChRs in the inflammation-related function of B lymphocytes, we studied the nicotinergic effects on the expression of inflammatory and apoptotic
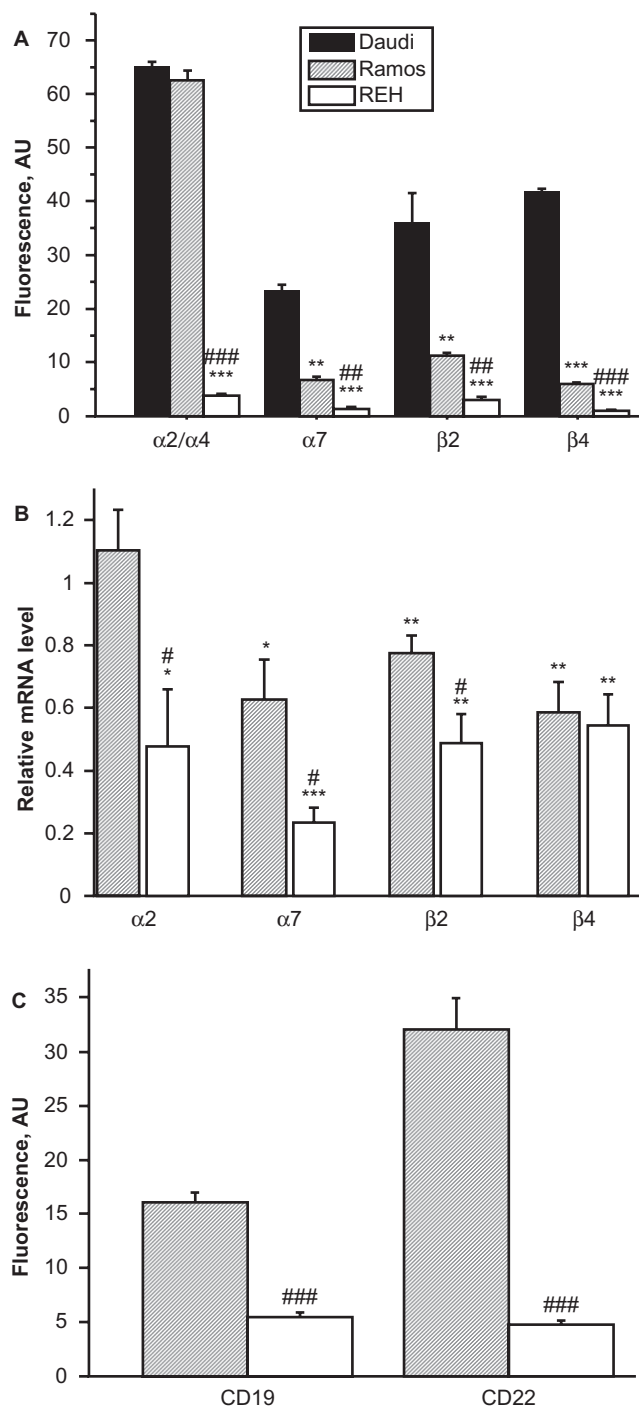
markers in Daudi cells activated with pansorbin. It is well established that activation of human B cells upregulates the inducible Cox-2 isoenzyme,[24] a marker of inflammation, and alters the expression/distribution of the apoptosis related proteins of the Bcl-2 family.[25,26] To activate B cells, we employed pansorbin, ie, heat-killed and fixed *Staphylococcus aureus* cells stimulating the mitogenic response of B lymphocytes.[27] First, we sought to confirm that pansorbin stimulation alters expression of the genes encoding the proinflammatory marker Cox-2, and the anti- and proapoptotic markers Bcl-2 and Bax, respectively, at the RNA and protein levels. As expected, stimulation of Daudi cells with 0.05% pansorbin caused a several fold increase of the Cox-2 mRNA and protein contents (Figure 2), indicating that pansorbin exhibits a pro-inflammatory action. The apoptosis-related effect of pansorbin was complex: it significantly ($P < 0.05$) upregulated the Bcl-2 mRNA and protein levels but also increased by almost fivefold the level of Bax protein, whereas the Bax mRNA level did not change compared to control ($P > 0.05$). These results indicated that the amounts of Bax and Bcl-2 proteins were regulated by pansorbin in different ways. An increase of Bcl-2 was caused by upregulated transcription, whereas that of Bax was induced at the post-transcriptional level. Since the increase of Bax protein was evidently stronger than that of Bcl-2, the net effect of pansorbin was proapoptotic.
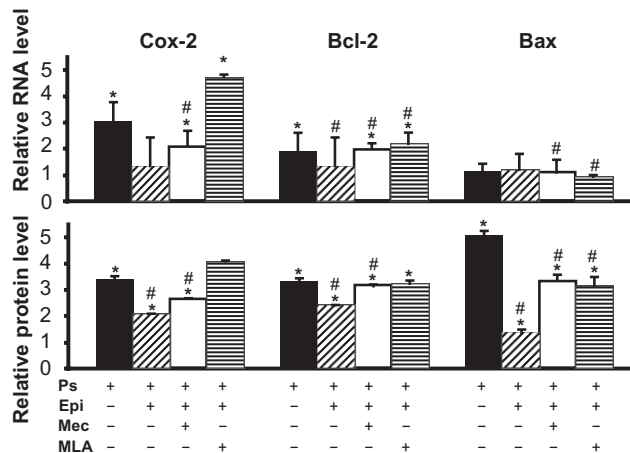
Figure 1 Expression of nAChR subunits and B cell differentiation markers in Daudi, Ramos, and REH cells. **A**) The Daudi, Ramos, and REH cells, 1×10^6 cells per tube in 50 μl of 1% BSA-containing PBS, were treated with rabbit α2/α4-, α7-, β2-, or β4-specific antibodies and the antibody binding was analyzed by flow cytometry as detailed in the Materials and methods section. **B**) qPCR analysis of equal amounts of cDNA from Daudi, Ramos and REH cells using the procedure described in Materials and methods. Results are fold of control values determined in Daudi cells, and taken as 1. **C**) Ramos and REH cells were stained with FITC-labeled anti-CD19 or PE-labeled anti-CD22 and analyzed by flow cytometry.
Notes: *$P < 0.005$, **$P < 0.005$, and ***$P < 0.0005$ compared to Daudi cells; #$P < 0.005$, ##$P < 0.005$, and ###$P < 0.0005$ compared to Ramos cells.
Abbreviations: AU, arbitrary units; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; nAChRs, nicotinic acetylcholine receptors; PBS, phosphate-buffered saline; PE, phycoerythrin; qPCR, quantitive polymerase chain reaction.

Figure 2 Nicotinic effects on pansorbin-induced activation of Daudi cells. Daudi cells, 3×10^6 cells/well, were incubated for 16 hours in a humid, 5% CO_2 incubator in the culture medium containing 0.05% pansorbin (Ps) in the absence or presence of 1 μM epibatidine (Epi) ± 50 μM Mec or 100 nM MLA, after which the expression of the genes encoding Cox-2, Bcl-2, and Bax at the mRNA and protein levels was measured by qPCR and ICW, respectively, as detailed in Materials and methods. The results are expressed as fold of control values determined in intact Daudi cells taken as 1.
Notes: *$P < 0.05$ compared to control values; £$P < 0.05$ compared to pansorbin given alone.
Abbreviations: ICW, in-cell Western blotting; Mec, mecamylamine; MLA, methyllycaconitine; qPCR, quantitative polymerase chain reaction.

To elucidate the role of nAChRs in the proinflammatory and proapoptotic responses of Daudi cells, we exposed the cells to pansorbin in the presence of 1 µM epibatidine, a nonselective nicotinic agonist.[28,29] We used the 1 µM concentration of epibatidine to activate all nAChR subtypes expressed in Daudi cells. Epibatidine abolished the pansorbin-induced upregulation of all studied biomarkers (Figure 2). While Cox-2 expression was suppressed at both mRNA and protein levels, a significant ($P < 0.05$) inhibition of pansorbin-induced Bcl-2 and Bax upregulation was observed only at the protein level. Since the decrease of Bax protein was much stronger than that of Bcl-2, the net effects of epibatidine was antiapoptotic. These findings indicated that nicotinergic signaling in B lymphocytes is both anti-inflammatory and antiapoptotic.

To estimate the relative contribution of α7 nAChRs to the observed effects of epibatidine, we applied MLA, a competitive inhibitor of α7-containing nAChRs.[30] We also used Mec, a channel blocker that can inhibit α7 as well as non-α7 nAChR subtypes such as α2.[28] The effect of epibatidine on the pansorbin-induced elevation of Cox-2 was abolished completely by MLA and, to a lesser extent, by Mec (Figure 2). In contrast, both antagonists were equally efficient in reducing the inhibitory effect of epibatidine on the pansorbin-dependent increase of Bcl-2 and Bax in Daudi cells (Figure 2).

These findings suggested that the anti-inflammatory nicotinergic signaling in B lymphocyte-derived cells is mediated predominantly through the α7-containing nAChRs, whereas apoptotic events are regulated through both α7 and non-α7 nAChRs.

The nicotinergic effects on the Daudi cell plasmacytic differentiation

To determine the role of the nicotinergic stimulation on regulation of B lymphocyte proliferation, we exposed Daudi cells to 1 or 10 µM epibatidine and measured the MTT inclusion after 48 hours of incubation. No statistically significant changes could be detected (data not shown).

Next, we measured changes in the relative amounts of mRNAs and proteins of markers of activation/plasmacytic differentiation in the Daudi cells exposed to epibatidine ± Mec, to activate/inactivate all nAChRs, or AR-R17779 ± MLA, to selectively activate/inactivate α7 nAChR.[31,32] The exposed cells where used in the qPCR and ICW assays of CD38, CD138 and Bcl-6 that had been previously identified as sensitive biomarkers of the Daudi cell state.[33] In most cases, both epibatidine and AR-R17779 produced only moderate changes

on the gene expression (Figure 3). However, epibatidine significantly ($P < 0.05$) upregulated CD38 at the transcriptional level and CD138 and Bcl-6 at the translational levels. AR-R17779 significantly ($P < 0.05$) increased the protein levels of CD38 and CD138 (Figure 3). In both cases, the effect of epibatidine was abolished with Mec, and that of AR-R17779 by MLA. These results demonstrated a functional role of nAChRs in regulating Daudi cells differentiation and revealed distinct contribution of α7 and non-α7 nAChRs in this process.

Discussion

In this study, we used human B lymphocyte-derived cell lines to investigate the role of α7 and non-α7 nAChRs in regulation of B-cell involvement in inflammation. Among the three B-cell lines tested, we selected Daudi cells because they appeared to express on their cell surfaces the highest amounts of nAChRs with a relatively high ratio of α7 to non-α7 nAChRs. Comparison of Ramos and REH cells demonstrated that the nAChR expression increases with B-cell maturation. These findings suggested that signaling through the nicotinic arm of acetylcholine regulatory axis is critical for maturation of B lymphocytes. This is in accord with our previously published data on mouse B lymphocytes.[9]

We showed for the first time that the nAChRs expressed by Daudi cells are coupled to an anti-inflammatory function, as nAChR activation with epibatidine inhibited pansorbin-induced upregulated Cox-2 expression.
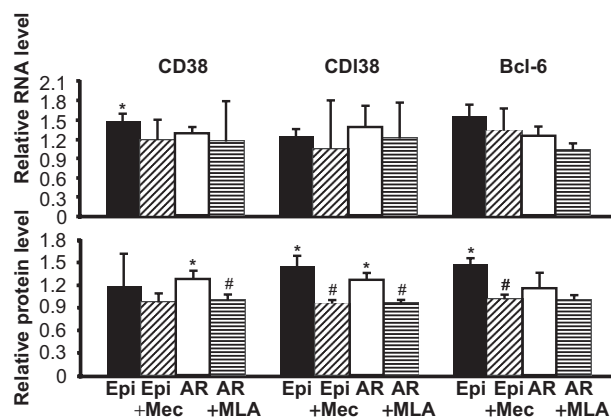

Figure 3 Nicotinic effects on the expression of differentiation markers in Daudi cells. Daudi cells, 3×10^6 cells/well, were incubated with 1 µM epibatidine (Epi) ± 50 µM Mec or 100 µM AR-R17779 (AR) ± 100 nM MLA, after which the expression of the genes encoding CD38, CD138, and Bcl-6 at the mRNA and protein levels was measured by qPCR and ICW, respectively, as detailed in the Materials and methods. The results are expressed as fold of control values determined in intact Daudi cells.
Notes: *$P < 0.05$ compared to control values; €$P < 0.05$ compared to a relevant agonist given alone.
Abbreviations: ICW, in-cell Western blotting; Mec, mecamylamine; MLA, methyllycaconitine; qPCR, quantitive polymerase chain reaction.

In keeping with the notion that α7 is coupled to suppression of inflammation,[6,14,22,23] the α7 selective antagonist MLA was more efficient than the nonselective antagonist Mec in abolishing the suppressive action of epibatidine on Cox-2 upregulation. We cannot ignore a possibility that some of the MLA effects were mediated by α9-made nAChR, but its expression in Daudi cells remains to be elucidated. Our data seem to disagree with the reports that nAChR signaling is pro-inflammatory in lymphocytes.[34,35] However, in contrast to the anti-inflammatory action of α7 nAChR, the proinflammatory cascade is believed to be activated through the pathways coupled by other nAChRs.[36]

The nicotinergic stimulation also considerably altered the pansorbin-induced expression of the genes regulating apoptosis of Daudi cells. as could be expected due to the fact that cellular processes mediating lymphocyte activation, inflammation, and cell death are interrelated. Since epibatidine abolished the elevation of Bax over Bcl-2 by pansorbin, and since both Mec and MLA antagonized epibatidine effects equally efficiently, we believe that the net nicotinergic effect was antiapoptotic in Daudi cells, and that both α7 and non-α7 nAChRs were involved. This is in keeping with a previous report that the β2- and α7-knockout mice show increased numbers of apoptotic B cells within the bone marrow.[9] The nAChR activation, however, did not affect Daudi cell proliferation, which may be explained by a strong proliferation potential of malignant cells.

The expression of cell activation/differentiation markers, such as CD38, CD138, and Bcl-6, was also sensitive to regulation through nAChRs. Stimulation of Daudi cells with the α7 agonist AR-R17779 upregulated expression of CD38 and CD138, but had no effect of Bcl-6 that, however, could be elevated by epibatidine. These observations highlighted a differential role of α7 and non-α7 nAChRs in cholinergic control of activation/plasmacytic differentiation of Daudi cells. CD38 is an ADP-ribosyl cyclase which also functions in cell adhesion, signal transduction and calcium signaling. It is considered as a marker of chronic B lymphocyte leukaemia and its receptor functions are connected to increased proliferative potential of B lymphocytes.[37] CD138 is a transmembrane (type I) heparan sulfate proteoglycan which is considered a marker of B lymphocyte plasmacytic differentiation.[38] BCL6 acts as a sequence-specific repressor of transcription, and its concomitant downregulation is required for completion of the plasma cell differentiation program.[39]

In conclusion, results of the present study emphasized an important role of the acetylcholine regulatory axis in B cell development and function. The obtained data demonstrated that nAChR signaling interferes with intracellular pathways triggered by B cell activation. Both α7 and non-α7 nAChRs are involved but fulfill different functions. Activation of distinct nAChR subtypes during B-cell development may exhibit differential effects on B-cell survival/activity. The unique coupling to regulation of a distinct cell function of nAChR subtypes expressed at different stages of B-cell development can diversify the immunoregulatory effects of the auto/paracrine cytotransmitter acetylcholine allowing it to coordinate the immune response to a specific environmental stimulus. Further elucidation of the acetylcholine receptor subtypes coupled to regulation of specific B cell functions will help in developing novel immunomodulatory therapies with nicotinic drugs allowing selective immunocorrection.

Acknowledgments

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