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Nicotinic receptors regulate B lymphocyte activation and immune response

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Abstract

The presence of nicotinic acetylcholine receptors (nicotinic receptors) composed of either α7 or α4 and β2 subunits is revealed in B lymphocytes by means of radioligand binding assay and Cell ELISA. Mouse B lymphocytes contained 12,200±3200 of epibatidine-binding sites and 3130±750 of α-Bungarotoxin-binding sites per cell. Mice lacking nicotinic receptor subunits α4, β2 or α7 had less serum IgG and IgG-producing cells in the spleen, but showed stronger immune response to both protein antigen in vivo and CD40-specific antibody in vitro than wild-type mice. Anti-CD40-stimulated proliferation of B lymphocytes from β2 knockout, but not wild-type mice was inhibited with nicotine. Our results indicate that signalling through nicotinic receptors affects both the pre-immune state and activation of B lymphocytes in the immune response, possibly via CD40-dependent pathway. This could contribute to immune depression found in tobacco smokers.

Keywords: Nicotinic receptor; Lymphocyte; Immune response

1. Introduction

Two main types of neuronal nicotinic acetylcholine receptors with different pharmacological and kinetic properties are found in mammals: the homopentameric receptor α7, and the heteropentameric receptors composed of alpha and beta subunits (Lindstrom et al., 1998.). Both nicotinic receptor subtypes are well characterised in the brain (Paterson and Nordberg, 2000) and autonomic ganglia (Skok, 2002) where they modulate synaptic transmission. In addition to the central nervous system, nicotinic receptors are expressed in some non-excitable tissues such as skin (Grando, 1997), respiratory epithelium (Maus et al., 1998), vascular endothelium (Macklin et al., 1998) and immune organs (Mihovilovic et al., 1997).

In the immune system nicotinic receptors were shown to be of special interest. For example, in macrophages these receptors regulate the production of pro-inflammatory cytokines (Borovikova et al., 2000), and in T lymphocytes, impairment of cell activation was associated with nicotine treatment (Kalra et al., 2000). Interestingly it is known that lymphocytes synthesize acetylcholine (Rinner et al., 1998), which may therefore be an autocrine or paracrine functional regulator (Fujii, 2004). However, although nicotine affects B lymphocyte responses (Savage et al., 1991), the exact role of nicotinic receptors in B lymphocytes is unknown. Studies performed on B lymphocyte-derived cell lines have demonstrated that nicotinic receptors are well expressed in those cells (Sato et al., 1999; Lustig et al., 2001) and are involved in regulating cell proliferation and antibody production (Skok et al., 2003). In this study, we describe the presence of α4β2 and α7 nicotinic receptors in normal mouse B lymphocytes and show that they are implicated in both forming the pre-immune status and the regulation of antibody response, possibly, by affecting the expression of and/or signalling through co-stimulatory molecule CD40.

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2. Materials and methods

2.1. Animals

We used age-matched male wild-type and mutant mice with common C57BL/6J background. Three different knockout mice were used, lacking either α4 (Marubio et al., 1999), β2 (Picciotto et al., 1995) or α7 (Orr-Urtreger et al., 1997) nicotinic receptor subunits. The mice were kept in the animal facility of Pasteur Institute, Paris. They were housed in a quiet, temperature-controlled room (22–23 °C) under a 12-h light–day cycle, and were provided with water and dry food pellets ad libitum. All procedures conformed to the guidelines of the Centre National de la Recherche Scientifique.

2.2. ELISA, ELISPOT and Cell ELISA assays

For testing total immunoglobulins and antibody-secreting cells, the plates for ELISA (enzyme-linked immunosorbent assay) were coated with rabbit anti-mouse μ-chain-specific (for IgM) or Fcγ-fragment-specific (for IgG) antibodies and blocked with 0.5% gelatin. Mouse blood sera were applied to the plates in 1:1000 to 1:8000 dilutions. The standard curves were generated with purified mouse IgM or IgG. The bound immunoglobulins were revealed with peroxidase-conjugated goat anti-mouse IgM (μ-chain-specific) or IgG ($F(ab')_2$-specific) followed by *o*-phenylendiamine-containing substrate solution. For testing natural antibodies, the plates were coated with rat myosin, actin or tubulin (5 μg/ml) and further processed as described above.

Mouse splenocytes resuspended in RPMI 1640 medium and supplemented with 2% fetal bovine serum were placed in ELISA plates coated with anti-mouse IgM or IgG ($15–50 \times 10^4$ cells per 100 μl per well) and incubated for 4–5 h at 37 °C and 5% CO_2. The secreted immunoglobulins were revealed with anti-IgM-alkaline phosphatase and anti-IgG-alkaline phosphatase conjugates, followed by 5-bromo-4-chloro-3-indolyl-αD-mannopyranoside (BSIP)-containing substrate solution. The number of spots was visualized and counted with an inverted microscope.

For Cell ELISA, the sorted B lymphocytes (1×10^5 per well) were adsorbed to the wells of ELISA plates by evaporation from PBS (phosphate-buffered saline) overnight at 37 °C and were fixed with methanol. The wells were blocked with 1% bovine serum albumin and incubated with biotinylated nicotinic receptor subunit-specific antibodies (Skok et al., 1999; Koval et al., 2004) (5 μg/ml) in Tween 20-containing PBS. The bound antibodies were revealed with avidin–peroxidase conjugate and *o*-phenylendiamine-containing substrate solution, the optical density being read at 490 nm.

2.3. Mouse cell preparation, sorting and fluorescent flow cytometry

Mice were sacrificed by cervical dislocation according to guidelines of the institutional committee. B lymphocytes were enriched from the splenocyte suspension by positive selection using magnetic beads-coupled either CD19-specific (for Cell ELISA and ligand binding assays) or CD45R (B220)-specific antibodies (for anti-CD40 stimulation) and AutoMACS device (Miltenyi Biotech) according to the manufacturer instructions. The sorted cell purity was checked by flow cytometry using double staining with fluorescein isothiocyanate-coupled B220-specific antibody (clone RA3-6B2, PharMingen) and R-phycoerythrin-coupled IgM-specific antibody (clone II-41, PharMingen). To check for CD40 expression, the splenocytes were stained with anti-B220-fluorescein isothiocyanate, anti-IgM-allophycocyanin and anti-CD40-biotin (clone HM40-3, PharMingen) followed by streptavidin-R-phycoerythrin (PharMingen). The cells were analysed with FACS-Calibur using the CELL-QUEST computer software (Beckton Dickinson).

2.4. Nicotinic receptor ligand binding study

Studies were performed as described (Skok et al., 2003). Briefly, magnetically enriched B lymphocytes ($2–2.5 \times 10^5$ per sample) were incubated with either 20 nM [^{125}I]-α-Bungarotoxin or 20 nM [^{3}H]-Epibatidine (Amersham) either in the absence (total binding) or in the presence (non-specific binding) of 2 mM nicotine, and washed with cold PBS on glass microfiber filters. Disintegrations on the filters were counted with either scintillation β-counter (for [^{3}H]-epibatidine) or a γ-counter (for [^{125}I]-α-Bungarotoxin). The

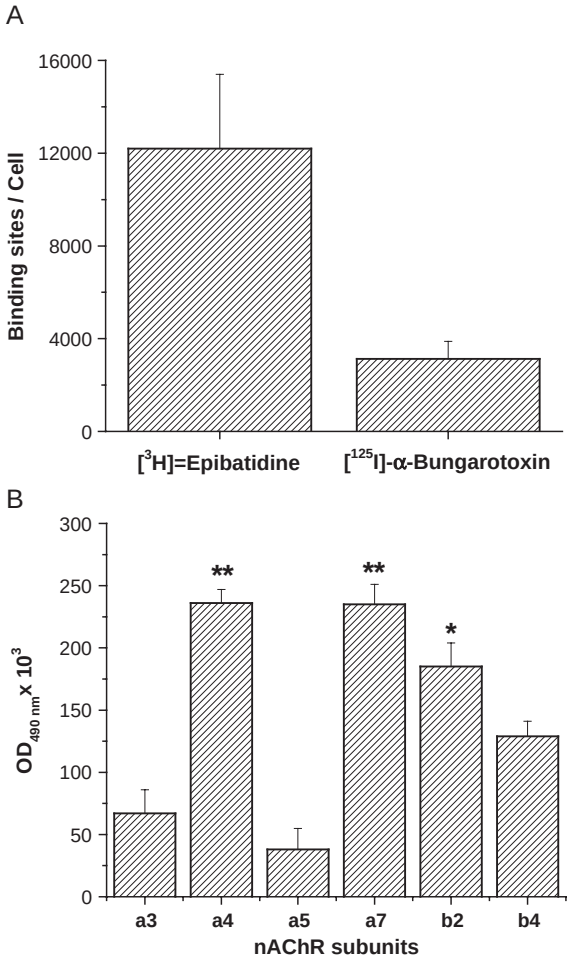

Fig. 1. Radioligand binding and Cell ELISA analysis of mouse CD19$^+$B lymphocytes. (A) [^{3}H]-Epibatidine (20 nM) and [^{125}I]-α-Bungarotoxin (20 nM) binding sites per cell. (B) The presence of nicotinic receptor subunits revealed using subunit-specific antibodies. Each column represents mean ± S.E.M. for cells of three mice (an average of three repeats for each mouse). The statistical significance is shown compared to α3-specific antibody binding. The binding of non-specific IgG is subtracted. B lymphocytes were purified by magnetic sorting from the spleens of C57BL/6J mice (see Materials and methods).

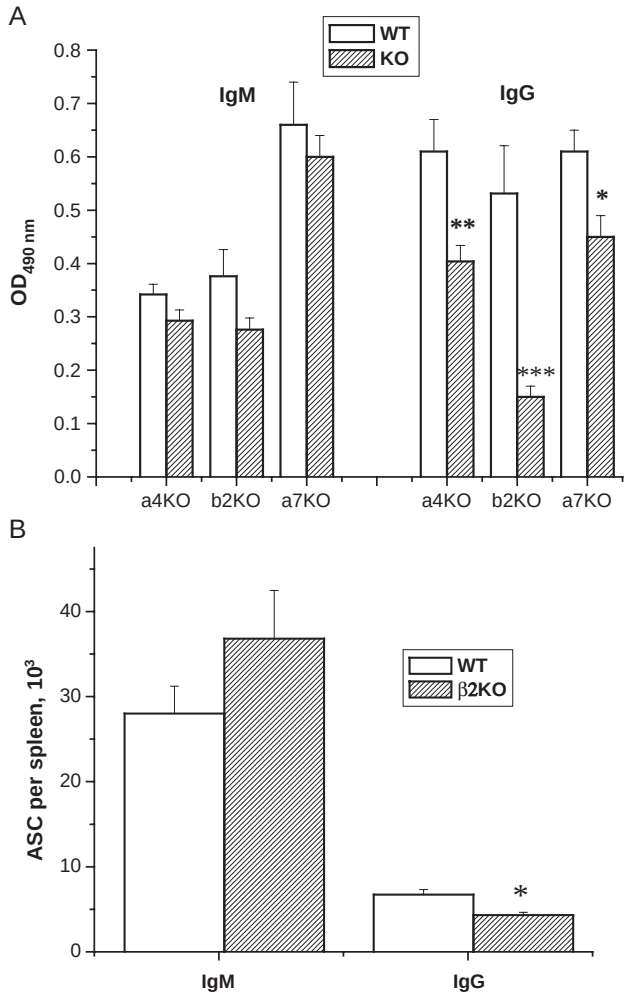

Fig. 2. Serum immunoglobulins in the blood of $\alpha4^{-/-}$, $\beta2^{-/-}$ and $\alpha7^{-/-}$ mice (A, $n=10$) and antibody-secreting cells (ASC) in the spleens of $\beta2^{-/-}$ mice (B, $n=4$) compared to their wild-type counterparts. Each column represents mean ± S.E.M. for the number of animals tested.

numbers of binding sites per cell (n) were calculated as $n = B \times N / c$, where B is the number of moles of ligand bound; N is Avogadro's number, and c is the number of cells per sample.

2.5. Immunization procedure and immune response evaluation

Wild type, $\alpha4^{-/-}$ and $\beta2^{-/-}$ mice were immunized subcutaneously with 100 μg of horse cytochrome *c* (Sigma, USA) emulsified in complete Freund's adjuvant. Every mouse was bled prior to immunization (day 0) and on days 16–19 post immunization. Six months later they were boosted and bled on days 0 and 15. Sera were tested by ELISA. The plates were coated with cytochrome *c* (10 μg/ml) and blocked with 0.5% gelatin. Sera were applied in 1:100 to 1:8000 dilutions. The bound antibodies were revealed with peroxidase-conjugated goat anti-mouse IgG ($F(ab')_2$-specific) and OPD-containing substrate solution, the optical density being read at 490 nm.

2.6. Proliferation assays

Either whole splenocytes or magnetically purified $B220^+$ B lymphocytes of the wild-type and $\beta2^{-/-}$mice, 1×10^6 per well, were stimulated with anti-CD40 antibody (PharMingen, clone HM40-3, 1:5000), in the presence or absence of nicotine. Whole splenocytes were also stimulated with anti-CD3ϵ antibody (PharMingen, clone 145-2C11, 1:2000). The antibody concentration was selected in preliminary experiments to be within the linear portion of the saturation curve. [^{3}H]-thymidine (1 μCi per well) was added in 24 h after stimulation and cell-incorporated radioactivity was measured 24 h later with the scintillation β-counter.

3. Results

The presence of native nicotinic receptors was investigated in $CD19^+B220^+IgM^+$ lymphocytes purified from the spleens of C57BL/6J mice by magnetic sorting procedure. These cells were first studied for heteromeric and homomeric nicotinic receptor, respectively, by [^{3}H]-Epibatidine and [^{125}I]-α-Bungarotoxin binding assays (Fig. 1A). The sorted B cells possessed 12,200 ± 3200 of

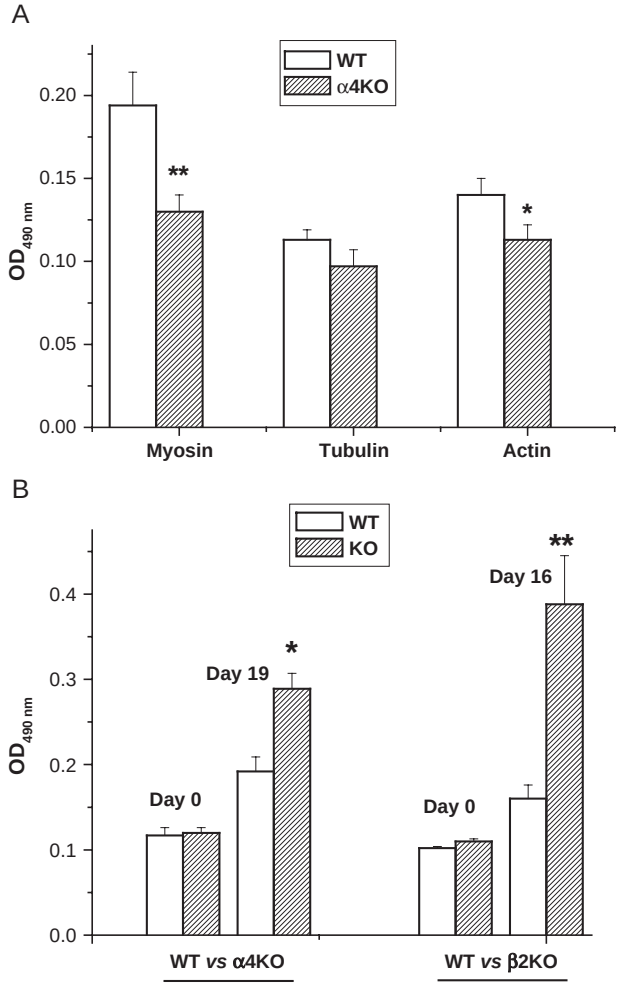

Fig. 3. Natural IgG antibodies in the pre-immune sera of $\alpha4^{-/-}$ and wild-type mice (A, $n=10$) and IgG response to cytochrome *c* in $\alpha4^{-/-}$ ($n=5$) and $\beta2^{-/-}$ ($n=9$) mice compared to their wild-type counterparts (B). Sera (1:1000) taken before (day 0) and after (days 16-19) primary immunization were analysed by ELISA. Each column represents mean ± S.E.M. for the number of animals tested.

epibatidine-binding sites and 3130±750 of α-Bungarotoxin-binding sites per cell (means and S.E.M of three independent experiments). Cell surface nicotinic receptor subtypes were additionally analysed by Cell ELISA with α and β nicotinic receptor subunit-specific antibodies generated by us previously and tested in autonomic ganglia neurons (Skok et al., 1999; Koval et al., 2004). As shown in Fig. 1B, specific labelling was observed for α4-, β2- and α7-specific antibodies.

Further experiments were conducted to elucidate the role of nicotinic receptors in antibody-producing B lymphocytes. Pre-immune levels of serum immunoglobulins and antibody immune responses were measured in wild-type and mice lacking α4, α7 or β2 nicotinic receptor subunits. As shown in Fig. 2A, all three types of knockout mice tested had significantly reduced amounts of serum IgG, but little change in IgM when compared to wild-type mice. In accordance with these results, the number of IgG- but not of IgM-secreting cells was reduced in the spleens of $\beta2^{-/-}$ mice (Fig. 2B). The level of pre-immune "natural" IgG antibodies able to bind actin and myosin, but not tubulin and cytochrome *c*, was significantly lower in $\alpha4^{-/-}$ than in the wild-type mice (Fig 3A). In contrast, the IgG response to horse cytochrome *c* was stronger in α4 and β2 knockout than in the wild-type mice (Fig. 3B). Six months after primary immunization, the $\beta2^{-/-}$ mice still had higher levels of cytochrome *c*-specific antibodies than the wild-type, while after the secondary immunization the difference was non-significant (data not shown).

Additionally, the B lymphocyte proliferative response to anti-CD40 antibody was studied in wild-type and $\beta2^{-/-}$ mice either in the presence or absence of nicotine. As shown in Fig. 4, B lymphocytes from $\beta2^{-/-}$ mice responded to anti-CD40 with slightly (about 20%), but significantly greater magnitude than those from wild-type control mice. This difference was observed in both total splenocytes (7 wild-type and 7 $\beta2^{-/-}$ mice) and magnetically purified B lymphocytes (3 wild-type and 3 $\beta2^{-/-}$ mice) stimulated with anti-CD40. Nicotine did not influence the CD40-induced proliferation of the wild-type cells, but inhibited that of β2 knockout by about 30%. The T lymphocyte proliferation stimulated with anti-CD3ϵ antibody was comparable between $\beta2^{-/-}$ and wild-type mice (data not shown).

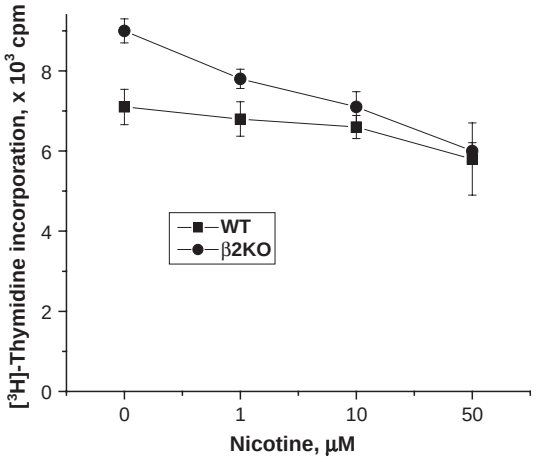

Fig. 4. Proliferation of splenic lymphocytes of the wild-type ($n=3$) and $\beta2^{-/-}$ ($n=3$) mice in response to anti-CD40 in the presence or absence of nicotine. Each point represents mean±S.E.M.

4. Discussion

The presence of nicotinic receptors in normal B lymphocytes was first revealed by radioligand binding assays (Fig. 1A). Epibatidine is known to bind heteromeric nicotinic receptors (Sihver et al., 1999), whereas α-Bungarotoxin is specific to both muscle-type and homomeric nicotinic receptors (Lindstrom et al., 1987; McLane et al., 1991; Couturier et al., 1990). Since no α-Bungarotoxin binding sites were found in splenocytes of mice lacking the α7 nicotinic receptor subunit (Cormier et al., 2004), it was suggested that α-Bungarotoxin bound α7 nicotinic receptors in wild-type B lymphocytes. This suggestion was further supported by Cell ELISA data (Fig. 1B). Altogether, our results indicated that the main nicotinic receptor subtypes found in B lymphocytes were homomeric α7 and heteromeric α4β2. The number of total epibatidine binding sites (12,200±3200 per cell) was similar to that found previously for myeloma X63-Ag8 cells (10,170±1100; Skok et al., 2003), while the number of surface α-Bungarotoxin binding sites (3130±750) was 2-fold less than that observed in myeloma cells (6730±370) supporting the hypothesis that homomeric α7 nicotinic receptors are involved in regulating myeloma and hybridoma cell proliferation (Skok et al., 2003). Compared to other non-excitable cells, the heteromeric nicotinic receptor levels in B lymphocytes is superior to the respiratory epithelial (630 of epibatidine-binding sites per cell; Maus et al., 1998), the vascular endothelial (990 of epibatidine-binding sites per cell; Macklin et al., 1998) and lymph node T cells (1500–1800 nicotine-binding sites per cell; Maslinski et al., 1992).

To elucidate the role of nicotinic receptors in B lymphocytes we tested the pre-immune antibody levels, the antibody immune response and B lymphocyte activation in mice lacking α4, α7 or β2 nicotinic receptor subunits and compared it to their wild-type counterparts. As shown in Fig. 2, the nicotinic receptor deficiency did not affect the basal amounts of IgM and IgM-producing cells, but the rate of cells, which switched from IgM to IgG production. In non-immunized animals, such a switch occurs in response to environmental antigens, mainly the bacterial polysaccharides (Janeway et al., 2001), and the number of IgG-producing cells strongly depends on the pre-immune repertoire of B cells. In our experiments, both $\alpha4^{-/-}$ and $\beta2^{-/-}$mice had similar levels of cytochrome *c*-specific IgG (Fig. 4), but less of total (Fig. 2), actin- and myosin-specific ($\alpha4^{-/-}$, Fig. 3) IgG compared to wild-type, suggesting that the lack of α4β2 nicotinic receptors resulted in the decrease of B cell repertoire.

The differences in both serum IgG levels and IgG-producing cell numbers between the wild-type and mutant mice were significant, although not of large magnitude. These data pointed out that the absence of a single nicotinic receptor subunit had only moderate effect on immune function. In order to track down this effect, both mutant and wild-type mice were immunized with a weak protein

antigen, horse cytochrome *c*. Due to a similarity with evolutionary conserved mouse cytochrome *c*, this antigen stimulates a special type of immune response which gives rise to IgG antibodies 2 weeks after the primary immunization (Skok and Komissarenko, 1995). In contrast to the wild-type mice, which responded poorly by days 16–19, both $\alpha4^{-/-}$ and $\beta2^{-/-}$mice had significantly stronger IgG immune response (Fig. 3).

To understand the apparent opposition in the pre-immune and post-immune effects of nicotinic receptor knockout versus wild-type mice, we considered that in contrast to the primarily T cell-independent reaction to environmental antigens, the T cell-dependent response to protein antigens requires CD40 signalling (Janeway, 2001). The direct measuring of CD40-stimulated B lymphocyte activation revealed that the lymphocytes of $\beta2^{-/-}$ mice were more sensitive to anti-CD40 than those of the wild-type mice. Surprisingly, nicotine diminished the anti-CD40 response of $\beta2^{-/-}$, but not wild-type mice (Fig. 4), demonstrating that its inhibitory effect was mediated through non-β2-containing receptors. The CD40-specific antibody binding to $IgM^{+}B220^{+}$ splenocytes (mean fluorescence intensity) was slightly higher in $\beta2^{-/-}$ and $\alpha7^{-/-}$ compared to the wild-type mice (11.4% and 8.4%, $p=0.0034$ and 0.043, respectively). These data indicated that nicotinic receptors affect signalling and expression of CD40, although the influence of α4β2 and α7 receptors is not identical. CD40 is the main co-stimulatory molecule of B lymphocytes. The connection of nicotinic receptor and CD40 was demonstrated recently in patients with persistent polyclonal B cell lymphocytosis (Loembe et al., 2001), which develops primarily in female smokers and is characterized by the inability of B lymphocytes to proliferate in response to anti-CD40.

CD40 functions in concert with the antigen-specific B cell receptor in the course of physiological B lymphocyte activation (Malapati and Pierce, 2001). We observed the non-significantly increased levels of surface IgM in $B220^{+}$ spleen cells of $\beta2^{-/-}$ and $\alpha7^{-/-}$ mice (data not shown) indicating that α4β2 and α7 nicotinic receptors may also affect B cell receptor expression or signalling. This notion correlates with the finding that chronic nicotine exposure impaired antigen-dependent signalling in B lymphocytes (Geng et al., 1995). Both B cell receptor and CD40 are critical for B lymphocyte selection during development. It may be hypothesized that the smaller pre-immune repertoire of nicotinic receptor-deficient mice is due to increased CD40/BCR signalling in the course of B lymphocyte development.

The suppressive effect of nicotine on the immune response, as well as the expression of nicotinic receptors in T lymphocytes is well documented (Sopori et al., 1998; Hallquist et al., 2000; Singh et al., 2000; Kalra et al., 2000). Indirectly, this is consistent with our data on increased antibody response in nicotinic receptor-deficient mice. Our results show for the first time that α4β2 and α7 nicotinic receptors are present in normal B lymphocytes and that the deficiency of those subunits affected both the pre-immune status of mice and their immune response. As such, the presented data indicate that nicotinic receptors are involved in B cell activation through CD40-mediated pathway. This finding raises a question of the source of acetylcholine which activates these receptors in vivo. Although acetylcholine released by the nerve terminals is quickly degraded by acetylcholineesterase, vagus nerve stimulation affects blood macrophages through cell-surface α7 nicotinic (Borovikova et al., 2000). As already mentioned, T lymphocytes produce endogenous acetylcholine (Rinner et al., 1998); therefore, signalling through nicotinic receptors may both mediate the neuro-immune interactions and be an additional pathway for the cross talk of B and T lymphocytes. The latter suggestion is indirectly supported by our finding that β2 nicotinic receptor deficiency affected B lymphocyte (CD40), but not T lymphocyte (CD3) proliferation. Furthermore, the rapid degradation of acetylcholine suggests that the B and T lymphocytes must be in close apposition for the nAChR-mediated effect to occur.

It should be noted that the effects observed in our experiments were significant, but rather small in magnitude (30–40%). Nevertheless, they correlated well with the changes observed in animals subjected to cigarette smoke (Sopori et al., 1989). This suggests signalling through nicotinic receptors is likely to exert a modulating, “tuning” effect on B lymphocyte-dependent immune processes.

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