Muscarinic Acetylcholine Receptors Modulate Interleukin-6 Production and Immunoglobulin Class Switching in Daudi Cells

Masato Mashimo, Marina Fujii, Natsumi Sakagawa, Yoshika Fukuda, Rika Imanaka, and Takeshi Fujii*

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Doshisha Women’s College of Liberal Arts;
97–1 Kodo, Kyotanabe, Kyoto 610–0395, Japan.
Received June 1, 2020; accepted September 25, 2020

INTRODUCTION

Immune cells, including T cells, B cells and macrophages, express such cholinergic system components as an acetylcholine (ACh)-synthesizing protein (choline acetyltransferase (ChAT)) and both muscarinic and nicotinic acetylcholine receptors (mAChRs and nAChRs, respectively). Moreover, in vivo and in vitro studies using cells and mice deficient in mAChR subtypes or nAChR subunits have provided evidence that signaling via both mAChRs and nAChRs impacts immune function. For example, after immunization, M1 and M5 mAChR double-deficient mice produce significantly less, total and antigen-specific immunoglobulin G (IgG) and interleukin (IL)-6 than do their wild-type counterparts. However, these mAChRs, but not nAChRs, mediated immunoglobulin class switching to IgG. This effect was blocked by scopolamine, a non-selective mAChR antagonist, and 4-diphenylacetoxy-N-methyl-piperidine methiodide (4-DAMP), a Gq/11-coupled M1, M3, M4 antagonist. In addition, IL-6 secretion was further enhanced following mAChR activation. Thus, Gq/11-coupled mAChRs expressed in B cells thus appear to contribute to IL-6 production and B cell maturation into IgG-producing plasma cells.

Key words muscarinic acetylcholine receptor; B cell; immunoglobulin class switching; interleukin-6

MATERIALS AND METHODS

Cell Culture Daudi cells (human Burkitt’s lymphoma, B cell leukemia) were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C under a humidified atmosphere with 5% CO2. Cells were cultured with 0.2% Pansorbin (heat-killed, formalin-fixed Staphylococcus aureus coated with protein A) upregulated expression of M1–M5 mAChRs and the a4 nAChR subunit. Under these conditions, mAChRs, but not nAChRs, mediated immunoglobulin class switching to IgG. This effect was blocked by scopolamine, a non-selective mAChR antagonist, and 4-diphenylacetoxy-N-methyl-piperidine methiodide (4-DAMP), a Gq/11-coupled M1, M3, M4 antagonist. In addition, IL-6 secretion was further enhanced following mAChR activation. Thus, Gq/11-coupled mAChRs expressed in B cells thus appear to contribute to IL-6 production and B cell maturation into IgG-producing plasma cells.

Enzyme-Linked Immunosorbent Assay (ELISA) Levels of IL-6 in culture supernatants were quantified using a sandwich ELISA. The capture antibody for IL-6 (2 μg/mL, MP5-20F3, BD Biosciences, Franklin Lakes, NJ, U.S.A.) was coated onto 96-well plates. Then after blocking with 0.5% bo-
Fig. 1. Pansorbin Induces mAChR and nAChR Gene Expression in Daudi Cells

Daudi cells exposed to Pansorbin (0.2%, 24 h) were subjected to real-time PCR using primers specific for M₁-M₅ mAChRs (A) and the α4, α7 and β2 nAChR subunits (B). GAPDH were used as an internal control to normalize the variability in expression levels. Bars depict means ± S.E.M. (n = 3). *p < 0.05, **p < 0.01.

Fig. 2. mAChR Activation Enhances Immunoglobulin Class Switching from IgM to IgG in Daudi Cells

A. Percentages of Daudi cells with surface expression of IgM and IgG in the absence or presence of nicotine and Oxo-M. Cells were exposed to nicotine (500 µM) or Oxo-M (300 µM) for 5d and then subjected to flow cytometry using FITC-conjugated anti-IgG and APC-conjugated anti-IgM. Bars represent means ± S.E.M. for at least three samples. B. Representative flow cytometry data showing surface expression of IgM (right) and IgG (left) in Daudi cells. Vehicle (filled gray), Pansorbin (black line), Pansorbin + nicotine (light gray line), Pansorbin + Oxo-M (gray line). C–E. Percentages of cells with surface expression of IgM and IgG in the presence Pansorbin plus an mAChR or nAChR agonist (Oxo-M, nicotine) with or without a corresponding antagonist (scopolamine, mecamylamine, DHβE, 4-DAMP, AFDX-383 (all 10 µM)). Bars depict means ± S.E.M. (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001.
vine serum albumin (BSA) in PBS containing 0.5% Tween 20, diluted samples and recombinant protein standards were added to the plates and incubated for 1 h at room temperature. They were then incubated for an additional 1 h with biotin-conjugated detection Abs (1 μg/mL MPS-32C11, BD Biosciences) at 37 °C and reacted with streptavidin-conjugated horseradish peroxidase, followed by o-phenylenediamine. The reaction was terminated by addition of 0.5 M H$_2$SO$_4$. The absorbance at 490 nm was then measured, and a graph was created using data from three samples.

**Flow Cytometry** To detect IgG and IgM, Daudi cells were stained using fluorescein isothiocyanate (FITC)-conjugated anti-IgG Ab (RM4.5, Thermo Fisher Scientific, Waltham, MA, U.S.A.) and APC-conjugated anti-IgM Ab (PC61.5, Thermo Fisher Scientific) in Hanks’ balanced salt solution supplemented with 0.1% BSA and 0.1% NaN$_3$, and subjected to flow cytometry (Cytomark, Brea, CA, U.S.A.). A gate was set on the lymphocytes using appropriate forward scatter and side scatter parameters. Isotype-matched FITC and APC-conjugated mouse IgG1 Abs were used as controls. The acquired data were analyzed using CytExpert (Beckman Coulter).

**Statistical Analysis** Data are presented as means ± standard error of the mean (S.E.M.). All experiments were repeated three times. Statistical analyses were performed using SigmaPlot (Systat Software Inc., San Jose, CA, U.S.A.). Differences between two groups were evaluated using Student’s t-test, and between three or more groups using one- or two-way ANOVA with post hoc Dunnett’s or Tukey’s test, respectively. Values of p < 0.05 were considered significant.

**RESULTS AND DISCUSSION**

### B Cell Activation Alters mAChR and nAChR Gene Expression in Daudi Cells
Several types of mAChRs and nAChRs are reportedly expressed in B cells.2,7) We investigated whether the expression levels of these receptors were changed by immune stimulation in Daudi human leukemic B cells. Pansorbin cells, which are heat-killed, formalin-fixed Staphylococcus aureus coated with protein A, were used to trigger B cell activation through binding to toll-like receptor 2 (TLR2).8) Stimulation with Pansorbin for 24 h significantly increased gene expression of the α1–M4 mAChR subtypes and the α4 nAChR subunit (Fig. 1).

#### mAChR Activation Enhances Pansorbin-Induced Immunoglobulin Class Switching from IgM to IgG
We used flow cytometry to investigate whether mAChRs and/or nAChRs are involved in immunoglobulin class switching in Daudi cells. Incubating Daudi cells for 5 d with nicotine or Oxo-M, a mAChR agonist, did not affect IgM content or induce IgG production (Fig. 2A). By contrast, Pansorbin increased IgG production and slightly decreased IgM content (Figs. 2B, C). Treatment with Pansorbin plus Oxo-M, but not nicotine, further enhanced IgG production (Figs. 2B, C). Scopolamine, a non-selective mAChR antagonist, eliminated the Oxo-M-induced increase in IgG production (Fig. 2D). By contrast, mecamylamine, a nAChR antagonist, and dihydro-β-erythroidine (DHβE), a α4/β2 nAChR antagonist, did not affect IgG production (Fig. 2E). In addition, 4-diphenylacetoxy-N-methyl-piperidine methiodide (4-DAMP), a G$_{q/11}$-coupled M$_1$, M$_2$, M$_3$, M$_4$ mAChR antagonist, but not ADFX-384, a G$_{i/o}$-coupled M$_2$, M$_3$, mAChR antagonist, suppressed Oxo-M-induced increases in IgG production in Daudi cells. M$_1$, M$_2$, M$_3$, M$_4$ mAChR agonist, suppressed Oxo-M-induced increases in IgG production in Daudi cells. These results indicate that G$_{q/11}$-coupled mAChR activation enhances immunoglobulin class switching.

#### mAChR Activation Enhances Pansorbin-Induced IL-6 Release
The cytokine IL-6 is secreted by several immune cell types, including B cells, and promotes differentiation of B cells into plasma cells.9) Treatment with Oxo-M promoted Pansorbin-induced IL-6 production in Daudi cells, and this effect was inhibited by pretreatment with scopolamine (Fig. 3).

Our findings summarized above indicate that some mAChR subtypes and nAChR subunits are upregulated upon B cell activation, and that activation of mAChRs, but not nAChRs, promotes immunoglobulin class switching to IgG as well as production and release IL-6. These findings are consistent with earlier observations from M$_1$/M$_4$ mAChR-deficient mice.6) IL-6 is required for B cell differentiation, and its production following TLR2 activation is regulated by several kinases and transcription factors, including extracellular signal-regulated kinase (ERK) and nuclear factor-kappaB (NFκB).10) M$_3$, M$_1$ and M$_4$ mAChRs are coupled to G$_{q/11}$ proteins, which activate phospholipase C (PLC)-β to signal through calcium and protein kinase C (PKC) signaling pathways. The fact that PKC can activate ERK signaling may provide a clue to the pathway via which G$_{q/11}$-coupled M$_1$, M$_3$, M$_4$ mAChR activation enhances IL-6 production, which in turn may promote differentiation into plasma cells and immunoglobulin class switching. M$_1$–M$_4$ mAChR gene expression was upregulated in response to Pansorbin. Determining which type of G$_{q/11}$-coupled mAChR mediates the response will be the aim of a future study.

Although Pansorbin increased expression of α4 subunit mRNA, nAChR activation does not appear to be involved in IgG production in Daudi cells. This finding differs from earlier observations in a7 nAChR-deficient mice.9) Our recent study indicates that a7 nAChRs expressed in antigen-presenting cells such as macrophages and dendritic cells interferes with their antigen presentation, thereby inhibiting T cell differentiation, including differentiation into T helper 2 (Th2) cells.9) Those findings together with the results of our present study suggest that a7 nAChRs expressed in B cells do not affect B cell differentiation or immunoglobulin class switching. By contrast, a7 nAChRs expressed in antigen presenting cells appear to suppress both T cell differentiation into Th2 cells and B cell differentiation into plasma cells as well as IgG secretion.
Acknowledgments  This study was supported by Grants-in-Aid for Scientific Research (C) (18K06903) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by Individual Research Grants from the Doshisha Women’s College of Liberal arts (TF).

Conflict of Interest  The authors declare no conflict of interest.

REFERENCES

1) Fujii T, Mashimo M, Moriwaki Y, Misawa H, Ono S, Horiguchi K, Kawashima K. Physiological functions of the cholinergic system in immune cells. J. Pharmacol. Sci., 134, 1–21 (2017).

2) Fujii T, Mashimo M, Moriwaki Y, Misawa H, Ono S, Horiguchi K, Kawashima K. Expression and function of the cholinergic system in immune cells. Front. Immunol., 8, 1085 (2017).

3) Skok M, Graillot R, Changeux JP. Nicotinic receptors regulate B lymphocyte activation and immune response. Eur. J. Pharmacol., 517, 246–251 (2005).

4) Mashimo M, Komori M, Matsu YY, Murase MX, Fujii T, Take-shima S, Okuyama H, Ono S, Moriwaki Y, Misawa H, Kawashima K. Distinct roles of alpha7 nAChRs in antigen-presenting cells and CD4+ T cells in the regulation of T cell differentiation. Front. Immunol., 10, 1102 (2019).

5) Fujii YX, Fujigaya H, Moriwaki Y, Misawa H, Kasahara T, Grando SA, Kawashima K. Enhanced serum antigen-specific IgG1 and proinflammatory cytokine production in nicotinic acetylcholine receptor alpha7 subunit gene knockout mice. J. Neuroimmunol., 189, 69–74 (2007).

6) Fujii YX, Tashiro A, Arimoto K, Fujigaya H, Moriwaki Y, Misawa H, Fujii T, Matsu M, Kasahara T, Kawashima K. Diminished antigen-specific IgG1 and interleukin-6 production and acetylcholinesterase expression in combined M1 and M5 muscarinic acetylcholine receptor knockout mice. J. Neuroimmunol., 188, 80–85 (2007).

7) Hainke S, Wildmann J, Del Rey A. Deletion of muscarinic type I acetylcholine receptors alters splenic lymphocyte functions and splenic noradrenaline concentration. Int. Immunopharmacol., 29, 135–142 (2015).

8) Bekeredjian-Ding I, Inamura S, Giese T, Moll H, Endres S, Sugi A, Zahringer U, Hartmann G. Staphylococcus aureus protein A triggers T cell-independent B cell proliferation by sensitizing B cells for TLR2 ligands. J. Immunol., 178, 2803–2812 (2007).

9) Hirano T, Taka T, Nakano N, Yasukawa K, Kashiwamura S, Shimizu K, Nakajima K, Pyun KH, Kishimoto T. Purification to homogeneity and characterization of human B-cell differentiation factor (BCDF or BSF-2). Proc. Natl. Acad. Sci. U.S.A., 82, 5490–5494 (1985).

10) Kuriakose S, Onylagha C, Singh R, Olayinka-Adefemi F, Jia P, Uzoma JE. TLR-2 and MyD88-dependent activation of MAPK and STAT proteins regulates proinflammatory cytokine response and immunity to experimental trypanosoma congolense infection. Front. Immunol., 10, 2673 (2019).

11) Resende RR, Adhikari A. Cholinergic receptor pathways involved in apoptosis, cell proliferation and neuronal differentiation. Cell Commun. Signal., 7, 20 (2009).