Note

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

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Received June 1, 2020; accepted September 25, 2020

B cells express muscarinic and nicotinic acetylcholine receptors (mAChRs and nAChRs, respectively). Following immunization with ovalbumin, serum immunoglobulin G (IgG) and interleukin (IL)-6 levels were lower in M_1 and M_5 mAChR double-deficient mice and higher in $\alpha 7$ nAChR-deficient mice than in wild-type mice. This suggests mAChRs participate in the cytokine production involved in B cell differentiation into plasma cells, which induces immunoglobulin class switching from IgM to IgG. However, because these results were obtained with conventional knockout mice, in which all cells in the body were affected, the specific roles of these receptors expressed in B cells remains unclear. In the present study, Daudi B lymphoblast cells were used to investigate the specific roles of mAChRs and nAChR in B cells. Stimulating Daudi cells using Pansorbin cells (heat-killed, formalin-fixed *Staphylococcus aureus* coated with protein A) upregulated expression of M_1 – M_4 mAChRs and the $\alpha 4$ 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 $G_{q/11}$ -coupled M_1 , M_3 , M_5 antagonist. In addition, IL-6 secretion was further enhanced following mAChR activation. Thus, $G_{q/11}$ -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

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 (mAChR and nAChR, respectively).^{1–3} 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.⁴ For example, after immunization, M_1 and M_5 mAChR double-deficient mice produce significantly less, while $\alpha 7$ nAChR-deficient mice produce significantly more, total and antigen-specific immunoglobulin G (IgG)₁ and interleukin (IL)-6 than do their wild-type counterparts.^{5,6} IgM production, by contrast, is unaffected. This suggests that both mAChRs and nAChRs affect B cell function by regulating IL-6 production, which in turn leads to modulation of antibody class switching from IgM to IgG. However, these receptors are not involved in the initial generation of the antibody response. But because the results summarized above were obtained using conventional knockout mice, the affected AChRs would be eliminated from cells throughout the body, making the specific role of AChRs expressed in B cells unclear. In the present study, therefore, we investigated whether AChRs expressed in Daudi cells, a leukemic B cell line, are directly involved in immunoglobulin and cytokine production.

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 μ g/mL streptomycin at 37°C under a humidified atmosphere with 5% CO₂. Cells were cultured with 0.2% Pansorbin for 24 h to detect mAChR and nAChR gene expression and for 5 d (restimulation with Pansorbin plus an AChR agonist with or without a corresponding antagonist after 3 d) to induce IgG production.

Real-Time PCR Total RNAs were extracted from Daudi cells using Sepasol RNA II Super (Nacalai Tesque, Kyoto, Japan), and cDNAs were prepared by reverse transcription using a Prime Script RT reagent Kit (TaKaRa Bio, Shiga, Japan) in a S1000 Thermal Cycler (Bio-rad, Hercules, CA, U.S.A.). Real-time PCR analysis was conducted using CYBR premix EX taq, FAM-labeled probes, and predesigned primers (TaKaRa Bio) with a Thermal Cycler Dice Real Time System (TaKaRa Bio). The catalog numbers of predesigned primers were as follows: M_1 mAChR, HA229529; M_2 mAChR, HA243585; M_3 mAChR, HA229529; M_4 mAChR, HA221666; M_5 mAChR, HA274938; $\alpha 4$ nAChR, HA188413; $\alpha 7$ nAChR, HA164722; $\beta 2$ nAChR, HA100887; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), HA067812.

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 μ g/mL, MP5-20F3, BD Biosciences, Franklin Lakes, NJ, U.S.A.) was coated onto 96-well plates. Then after blocking with 0.5% bo-

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Fig. 1. Pansorbin Induces mAChR and nAChR Gene Expression in Daudi Cells

Daudi cells exposed to Pansorbin (0.2%, 24h) 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 ($1 \mu\text{g/mL}$ MP5-32C11, BD Biosciences) at 37°C and reacted with streptavidin-conjugated horseradish peroxidase, followed by *o*-phenylenediamine. The reaction was terminated by addition of $0.5 \text{ M H}_2\text{SO}_4$. 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 (CytoFLEX, Beckman Coulter, 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 $\pm$ 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 M_1 – M_4 mAChR subtypes and the α_4 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 (DH β E), a $\alpha 4\beta 2$ nAChR antagonist, did not affect IgG production (Fig. 2E). In addition, 4-diphenylacetoxy-*N*-methyl-piperidine methiodide (4-DAMP), a $\text{G}_{q/11}$ -coupled M_1 , M_3 , M_5 mAChR antagonist, but not ADFX-384, a $\text{G}_{i/o}$ -coupled

Fig. 3. mAChR Activation Enhances IL-6 Release from Daudi Cells

IL-6 released from Daudi cells was measured using an ELISA after 5 d exposure to Pansorbin (0.2%) with and without Oxo-M ($300 \mu\text{M}$) or Oxo-M + scopolamine ($10 \mu\text{M}$). Cells were pretreated with scopolamine for 30 min before adding to Oxo-M. Bars depict means $\pm$ S.E.M. ($n = 3$). * $p < 0.05$.

M_2 , M_4 mAChR antagonist, suppressed Oxo-M-induced increases in IgG production in Daudi cells. These results indicate that $\text{G}_{q/11}$ -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_5 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 (NF κ B).¹⁰⁾ M_1 , M_3 and M_5 mAChRs are coupled to $\text{G}_{q/11}$ 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 signaling¹¹⁾ may provide a clue to the pathway *via* which $\text{G}_{q/11}$ -coupled M_1 , M_3 , M_5 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 $\text{G}_{q/11}$ -coupled mAChR mediates the response will be the aim of a future study.

Although Pansorbin increased expression of $\alpha 4$ subunit mRNA, nAChR activation does not appear to be involved in IgG production in Daudi cells. This finding differs from earlier observations in $\alpha 7$ nAChR-deficient mice.⁵⁾ Our recent study indicates that $\alpha 7$ 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.⁴⁾ Those findings together with the results of our present study suggest that $\alpha 7$ nAChRs expressed in B cells do not affect B cell differentiation or immunoglobulin class switching. By contrast, $\alpha 7$ 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.

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