ORIGINAL ARTICLE

Roles of dopamine receptor subtypes in mediating modulation of T lymphocyte function

Yan Huang [1], Ao-Wang Qiu [2], Yu-Ping Peng [1], Yan Liu [1], Hui-Wei Huang [1], Yi-Hua Qiu [1]

1 Department of Physiology, School of Medicine, Nantong University, Nantong, P. R. China
2 The First Clinical Medical College of Nanjing Medical University, Nanjing, P. R. China

Correspondence to: Yu-Ping Peng,
Department of Physiology, School of Medicine, Nantong University
19 Qixiu Road, Nantong 226001, P. R. China.
tel: +86-513-85051722; fax: +86-513-85051714; e-mail: yppeng@ntu.edu.cn

Submitted: 2010-11-11 Accepted: 2010-11-20 Published online: 2011-01-09

Key words: **dopamine D1-like receptors; dopamine D2-like receptors; T lymphocytes; cAMP; CREB**

Neuroendocrinol Lett 2010; **31**(6):782–791 **PMID:** 21196914 NEL310610A11

Abstract

OBJECTIVE: Dopamine exists in the immune system and has obvious immunomodulating action. However, receptor mechanism underlying the dopamine immunomodulation remains to be clarified. In the present study, we provide the evidence for existence of dopamine receptor subtypes in T lymphocytes and show the roles of the receptors and the receptor-coupled signaling in mediating the dopamine immunomodulation.

METHODS: The purified T lymphocytes from the mesenteric lymph nodes of mice were detected for expressions of all five subtypes of dopamine receptor mRNAs by reverse transcription-polymerase chain reaction. Lymphocyte proliferation and production of interferon-γ (IFN-γ) and interleukin-4 (IL-4) in response to concanavalin A (Con A) were measured by colorimetric methyl-thiazole-tetrazolium assay and cytometric bead array, respectively, after the cells were exposed to dopamine D1-like or D2-like receptor agonists and antagonists. Meanwhile, content of cAMP and phosphorylation of cAMP-response element-binding (CREB) in the lymphocytes were examined by ^{125}I-cAMP radioimmunoassay and Western blot assay, respectively.

RESULTS: T lymphocytes expressed all the five subtypes of dopamine receptor mRNAs, i.e., D1, D2, D3, D4 and D5 receptors. SKF38393, an agonist of dopamine D1-like receptors (D1 and D5 receptors) only reduced the IFN-γ production, but did not significantly affect the proliferative response, IL-4 production, cAMP content or CREB activation of the lymphocytes. The SKF38393-induced decrease in IFN-γ level was blocked by the D1-like receptor antagonist SCH23390. Quinpirole, an agonist of dopamine D2-like receptors (D2, D3 and D4 receptors) attenuated the lymphocyte proliferation to Con A, and decreased the IFN-γ but increased the IL-4 production. Meanwhile, the quinpirole diminished the cAMP content and the phosphorylated CREB level in the lymphocytes. All the quinpirole-induced changes were reversed by dopamine D2-like receptor antagonist haloperidol.

CONCLUSIONS: Five dopamine receptor subtypes of the two families, D1-like and D2-like receptors, exist on T lymphocytes of mice. Of the two families, D2-like receptors are more important in mediating modulation of T cell function than D1-like receptors. D2-like receptors are involved in suppression of T helper 1 (Th1) cell function and enhancement of Th2 cell function through negative link to cAMP-CREB pathway.

INTRODUCTION

The presence of various receptors for neurotransmitters and hormones on immunocytes provides a substantial basis for the neural and endocrine regulation of immune cell function (Yin *et al.* 2006; Sarchielli *et al.* 2007; Levite 2008). It has been well known that β-adrenoreceptors (β-ARs) exist on lymphocytes and they mediate the modulation of lymphocyte function (Maestroni 2006; Pesić *et al.* 2007). Our recent work has revealed that T lymphocytes express α_1-ARs and α_2-ARs, and activation of the α_2-ARs suppresses T lymphocyte proliferation and cytokine production *in vitro* (Bao *et al.* 2007).

Dopamine exists in the immune system. It derives from endocrine glands, which release dopamine to immunocytes via blood stream, nerve terminals innervating immune tissues (Pacheco *et al.* 2009). Particularly, *in vitro* and *in vivo* studies have demonstrated that apart from its hemodynamic action dopamine can modulate immune responses (Beck *et al.* 2005). Several *in vitro* experiments on the effects of dopamine on murine and human lymphocytes have shown significant inhibition of lymphocyte proliferation, decrease in the synthesis of pro-inflammatory and increase in the synthesis of anti-inflammatory mediators (Cook-Mills *et al.* 1995; Offen *et al.* 1995; Josefsson *et al.* 1996; Bergquist *et al.* 1997; Basu *et al.* 2000; Beck *et al.* 2005). Dopamine-induced inhibition of T cell clone, CTLL-2 and B cell lymphoma, B9 cells, strongly indicates direct inhibitory property of dopamine on these cells (Josefsson *et al.* 1996). Activities of both resting, activated human T cells and antibody production could be suppressed significantly *in vitro* by a dopamine receptor agonist, bromocriptine (Morikawa *et al.* 1993, 1994). The involvement of dopamine receptors in the dopamine-mediated proliferation inhibition of lymphocytes is shown a possibility underlying mechanisms of dopamine-induced cell proliferation.

Dopamine receptors have been isolated, characterized and subdivided into two families, D1-like and D2-like receptors, on the basis of their biochemical and pharmacological properties (Vallone *et al.* 2000). The dopamine D1-like receptor family comprises D1 receptors (D1R) and D5R, and the D2-like receptor family includes D2R, D3R and D4R (Yao *et al.* 2008). In lymphocytes, the expression or presence of the different subtypes of dopamine receptors is not fully clear. Using flow cytometric techniques to identify dopamine receptors with subtype-specific antibodies, Mckenna *et al.* (2002) have indicated that of the D1-like receptor family, only D5 is detected, and of the D2-like receptor family all dopamine receptors are detected. T lymphocytes and monocytes have low expression of dopamine receptors, whereas neutrophils and eosinophils have moderate expression. B cells and NK cells have higher and more consistent expression. Dopamine receptors D3 and D5 are found in most individuals whereas D2 and D4 have more variable expression. D1 is never found. In agreement with the reports from Mckenna *et al.*, Ricci *et al.* (1999) present that dopamine D5 receptor is the only dopamine D1-like receptor subtype expressed by human peripheral blood lymphocytes. Molecular biological methods demonstrate the expression of D3, D4 and D5 receptors in human circulating lymphocytes (Bondy *et al.* 1996). However, unlike human lymphocytes, rat lymphocytes have the expression of D1 receptor mRNA, besides the expressions of D3 and D5 receptors (Caronti *et al.* 1998). These findings imply that the expression or presence of the different subtypes of dopamine receptors in lymphocytes varies widely with the different species and research methods. This is therefore worthy of being further explored.

The dopamine D1-like and D2-like receptors are often coupled to stimulation and inhibition of intracellular cAMP production respectively (Sibley *et al.* 1993). The D1-like receptors are positive regulator of intracellular cAMP levels (Monsma *et al.* 1990; Sunahara *et al.* 1991; Jackson *et al.* 1994). Their stimulation results in an activation of protein kinase A (PKA). PKA, in turn, phosphorylates cytoplasmic and nuclear proteins, and regulates ion channel function and gene expression (Choi *et al.* 1993; Hofmann *et al.* 1994; Rohr *et al.* 1999; Beck *et al.* 2004). The D2-like receptors are negatively coupled to this cAMP/PKA-dependent signaling (Vallone *et al.* 2000; Yao *et al.* 2008). The cAMP-induced gene expression is mediated by cAMP-response element-binding (CREB), a transcription factor, via its binding to a cAMP-response element in the gene promoter region (Montminy & Bilezikjian 1987). The activation of CREB and PKA can inhibit translocation of nuclear factor-κB, which is implicated in the immune modulation (Beck *et al.*, 2004). However, whether the signal-transduction molecules are linked to the different subtypes of dopamine receptors and involved in the modulation of lymphocyte function remains to be identified.

On the basis of the actualities, we hypothesized that the various subtypes of dopamine receptors are expressed by murine T lymphocytes and have the ability to modulate T cell function, which can be mediated by cAMP/PKA-CREB signal-transduction pathway coupled to the dopamine receptors. Thereby, in the current study, we firstly measured the expressions of all the five subtypes of dopamine receptors in T lymphocytes, and then explored their roles and relevant signaling molecules in the regulation of T cell function by use of receptor agonists and antagonists.

MATERIALS AND METHODS

Separation and purification of T lymphocytes

The mesenteric lymph nodes of mice were harvested by celiotomy. Lymphocytes were obtained by gently squeezing the mesenteric lymph nodes and then sus-

pended in RPMI 1640 (Sigma, USA). The final concentration of the lymphocytes in the complete culture medium was 3×10^7 to 4×10^7 cells/ml. T lymphocytes were purified by using the method of nylon wool column filtration (Greaves & Brown 1974; Bertram *et al.* 1997; Wohlera & Barnum 2009). Rinsed nylon fibers were loaded into syringe columns, which were sterilized. The nylon fibers in the columns were rinsed with RPMI 1640 containing 10% heat-inactivated calf serum at a flow rate of 2 ml/min. The lymphocyte suspension with RPMI 1640 was added to the columns containing the nylon fibers, which were incubated for 2 h at 37 °C. The columns were washed with RPMI 1640 containing 10% heat-inactivated calf serum at a flow rate of 2 ml/min and the eluted cell suspension was collected. The eluted cell suspension was resuspended in RPMI 1640 containing 10% heat-inactivated calf serum and again passed through the columns. Finally, the eluted lymphocyte suspension was examined for purification of the T cells by flow cytometry using fluorescein isothiocyanate (FITC) hamster anti-mouse CD3e (BD Biosciences, USA) (Fikri *et al.* 2000; Wohlera & Barnum 2009). Briefly, the cells were washed twice with ice-cold phosphate buffered saline containing 1% bovine serum albumin and then 10^6 cells per test were incubated on ice with the anti-CD3-FITC for 30 min. After washing, phenotypic analysis was performed on a FACS Calibur Flow Cytometer (BD Biosciences, USA). As shown in Figure 1, the percentage of the purified T lymphocytes (CD3+) in the mesenteric lymph node cells was 93.80 ± 0.53%, which was significantly higher than that, 74.10 ± 0.95%, of the non-purified T lymphocytes in the lymph node cells.

Reverse transcription-polymerase chain reaction (RT-PCR) for measurement of dopamine D1R-D5R mRNAs

Total RNA in the freshly isolated T lymphocytes was extracted with trizol reagent (Bio Basic Inc., Canada) according to the manufacturer's instructions. The concentration of RNA was quantitated by absorbance at 260 and 280 nm. Single-stranded cDNA was prepared using 5 μg of total RNA, 200 U MMLV reverse transcriptase (Fermentas, USA), 4 μl MMLV buffer (Promega, USA), 2 μl of 10 mM dNTP (Promega, USA), 1 μl oligo (dT) 18 (Amresco, USA) and 500 μU Rnasin (Amresco, USA) in 0.5 μl solution, which was incubated for 90 min at 42 °C. An amplification of the resulting cDNA sequence was carried out by using PCR. Oligonucleotide sequences of primers used in PCR for amplification of specific fragments were shown in Table 1. The conditions for the reaction were as follows: the tubes were incubated in a thermal cycler at 95 °C for 5 min, and 94 °C for 1 min, 60 °C (D1R, D2R and D3R) or 58 °C (D4R and D5R) for 1 min and 72 °C for 1 min, which were cycled for 35 times, and 72 °C for 5 min. The amplified products were resolved by electrophoresis in 1.5% agarose gel, stained with ethidium bromide.

Colorimetric methyl-thiazole-tetrazolium (MTT) assay of Con A-induced lymphocyte proliferation

The mesenteric lymph nodes of mice were harvested by celiotomy. Lymphocytes were obtained by gently squeezing the mesenteric lymph nodes and then suspended in RPMI 1640 (Sigma, USA) with 10% heat-inactivated calf serum, 2.5×10^{-2} M HEPES, 1×10^{-3} M sodium pyruvate, 5×10^{-5} M mercaptoethanol and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin). The final concentration of the lymphocytes in the complete culture medium was 1×10^6 cells/ml. The lymphocytes were incubated with D1-like receptor agonist SKF38393 or antagonist SCH23390, or with D2-like receptor agonist quinpirole or antagonist haloperidol. One hour later, concanavalin A (Con A, 5 μg/ml) was added to the cultures and they were incubated in an incubator (ESPEC BNA-311, Japan) with 5% CO_2 at 37 °C for 48 h. The control group was prepared as described above but without any drugs. MTT assay was quantitatively measured as described by Hansen *et al.* (1989). Namely, the cultures of lymphocytes were placed into the wells of a 96-well flat-bottom plate with MTT (Fluka, USA) solution of 5 mg/ml (10 μl MTT solution per 100 μl medium), followed by incubation with 5% CO_2 at 37 °C for 4–6 h. Twenty percent SDS that was made with 50% N, N-dimethylformamide was added to the cultures, which were incubated for 8 h in 5% CO_2 at 37 °C. Lastly, their optical density (OD) was read on a Universal Microplate Reader (ELX 800; Bio-Tek Instruments Inc., USA) using a test wavelength of 570 nm.

Cytometric bead array for determination of IFN-γ and IL-4 production

The preparation and activation of lymphocytes were the same as described in the Con A-induced lympho-

Tab. 1. Sequences of PCR primers.

Gene	Sense primer	Antisense primer	Product size (bp)	Accession No.
D1R	5′-TGTGACACGAGGTTGAGC-3′	5′-GGTGGTCTGGCAGTTCTT-3′	177	NM_010076
D2R	5′-CCATTGTCTGGGTCCTGT-3′	5′-TGCCCTTGAGTGGTGTCT-3′	258	NM_010077
D3R	5′-CTACGCCCTGTCCTACTGT-3′	5′-CCACCTGTCACCTCCAAG-3′	189	NM_007877
D4R	5′-GTGTTGGACGCCTTTCTTCG-3′	5′-GGGTTGAGGGCACTGTTGA-3	120	NM_007878
D5R	5′-CTGCGAGCATCCATCAAG-3	5′-CACAAGGGAAGCCAGTCC-3′	160	NM_013503

cyte proliferation. The supernatants of the lymphocyte cultures were collected and measured for the IFN-γ and IL-4 levels. The procedure was carried out according to the manufacturer's instruction (CBA™, BD Biosciences, USA). Briefly, the mixed mouse capture bead suspensions were added to the prepared supernatants of lymphocyte cultures. Phycoerythrin detection reagent was added to the mixtures, which was incubated for 2 h in dark at room temperature. The samples were washed with wash buffer and centrifuged at 200×g for 5 min. Test buffer was added to the deposits, which was analyzed on FACS Calibur Flow Cytometer (BD Biosciences, USA) using the supplied cytometer setup beads and the CellQuest™ Software (BD Biosciences, USA).

Radioimmunoassay of cAMP content

After incubation for 48 h, 1×10^6 lymphocytes were collected and subjected to centrifugation. The cell pellets were treated with 1 ml of 50 mM acetic acid (pH 4.75) and stored at –20 °C until analysis. The cells, broken by repeated freezing and thawing, were centrifuged at 3 000×g for 15 min at 4 °C. The supernatants were measured for cAMP level by radioimmunoassay using a commercially available kit (Beijing North Institute of Biological Technology, China). Briefly, portions of samples (100 µl/sample) were acetylated and then incubated with 100 µl of ^{125}I-cAMP (20,000 cpm/100 µl) and 100 µl of anti-cAMP antibody at 4 °C for 24 h. Rabbit serum (100 µl) and goat anti-rabbit IgG (100 µl) were added to the mixture, which was incubated for 12 h at 4 °C, and then centrifuged at 3 000×g and 4 °C for 20 min. Radioactivity of the deposits was measured by use of a multi-well gamma counter (Shanghai Rihuan Instrument Company, China).

Western blot analysis of phosphorylated CREB expression

Nuclear lysates were extracted from lymph node cells cultured with Con A for 48 h. Protein concentration was estimated by a modified Bradford assay, and 20 µg of protein was boiled for 5 min in 2×loading buffer. The protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidine difluoride membrane using a semi-dry transfer apparatus. After blocking nonspecific binding with 5% (w/v) nonfat dry milk, the membranes were probed with antibodies specific for cleaved phosphorylated CREB (1:1 000) and total CREB (1:1 000) (all from Cell Signaling Technology, USA) at room temperature for 2 h or at 4 °C overnight, followed by incubation with a fluorescently-conjugated secondary antibody for 1 h at room temperature and visualization by Odyssey laser scanning system (LI-COR Inc, USA). The molecular weight and relative quantity of the protein bands were determined by an image analysis system (Odyssey 2.1, LI-COR Inc, USA).

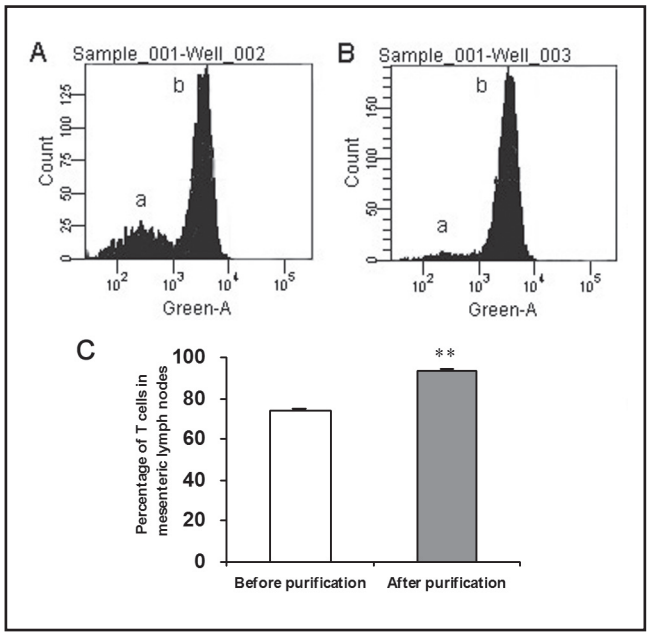

Fig. 1. Comparison of T lymphocyte purity. Lymphocytes from mesenteric lymph nodes of mice were isolated and purified using nylon wool columns. T lymphocytes were detected by flow cytometry using FITC hamster anti-mouse CD3e. (A) Lymphocyte subpopulations before T cell purification. (B) Lymphocyte subpopulations after T cell purification. In the (A) and (B), (a) is lymphocytes except CD3+ cells, and (b) is CD3+ lymphocytes, i.e., T lymphocytes. (C) Statistic chart of the data repeated for three times. **$p<0.01$, compared with the T lymphocytes before purification.

Statistical analysis

Data were expressed as means ± standard deviation. Statistical analyses were performed with the Statistics Package for Social Science (12.0, USA). The data were subjected to the one-way analysis of variance, followed by Student-Newman-Keul's test to compare the data of all groups relative to each other. Differences were considered statistically significant at $p<0.05$.

RESULTS

T lymphocytes express five subtypes of dopamine receptor mRNAs

In the separated and purified T lymphocytes, there were the expressions of all the five subtypes of dopamine receptor mRNAs, including D1 and D5 receptors (D1-like receptor family), and D2, D3 and D4 receptors (D2-like receptor family). The molecular weights were 177, 160, 258, 189 and 120 bp for the D1R, D5R, D2R, D3R and D4R, respectively. They were consistent with predicted sizes of PCR-amplified products for these dopamine receptor mRNAs (Figure 2).

Role of D1-like receptors in mediating modulation of T lymphocyte function

Lymphocytes were exposed to D1-like receptor agonist SKF38393 (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M), and

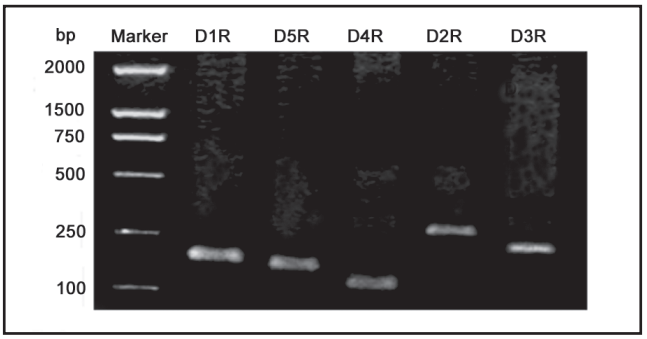

Fig. 2. Expressions of dopamine subtype receptor mRNAs in T lymphocytes. T lymphocytes of the mesenteric lymph nodes from mice were purified and detected for the dopamine subtype receptor mRNAs by using RT-PCR. In the electrophoretic bands, D1R and D5R are D1-like receptors, with the molecular weights of 177 and 160 bp, respectively, and D4R, D2R and D3R represent D2-like receptors, with the molecular weights of 120, 258 and 189 bp, respectively. The molecular weights of the bands are consistent with the predicted sizes of the PCR-amplified products.

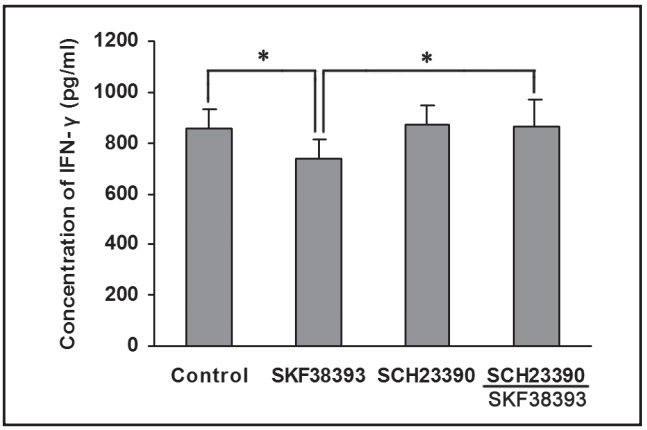

Fig. 3. Activation of D1-like receptors attenuates IFN-γ production by the Con A-stimulated lymphocytes. The mesenteric lymph node cells were cultured with Con A for 48 h in the presence of D1-like receptor agonist SKF38393 (10^{-8} M) or antagonist SCH23390 (10^{-7} M). In the culture supernatants, IFN-γ concentration was measured by cytometric bead array. *p<0.05.

stimulated by mitogen Con A for 48 h. The MTT OD values reflecting T cell proliferation were 1.04 ± 0.10, 1.04 ± 0.09, 1.00 ± 0.13, 1.04 ± 0.13 and 1.05 ± 0.12 for the five concentrations of SKF38393, respectively. They did not exhibit significant differences from 1.09 ± 0.08 of control lacking SKF38393 exposure.

Likewise, the SKF38393 (10^{-8} M) exposure did not significantly affect the production of IL-4 by the Con A-stimulated lymphocytes. The IL-4 concentration in the supernatants of lymphocyte cultures was 9.61 ± 1.83 vs. 9.70 ± 2.01 pg/ml (SKF38393 vs. control). However, the SKF38393 exposure markedly reduced IFN-γ level in the lymphocyte culture supernatants, showing that SKF38393 inhibits IFN-γ production by the Con A-stimulated lymphocytes. The dopamine D1-like receptor antagonist SCH23390 (10^{-7} M) blocked this effect of SKF38393 (Figure 3).

Activation of lymphocyte D1-like receptors does not influence cAMP content and phosphorylated CREB expression in the cells

Did neither the D1-like receptor agonist SKF38393 (10^{-8} M) nor the antagonist SCH23390 (10^{-7} M) change the intracellular cAMP content (data were not listed) or phosphorylated CREB expression in the Con A-activated lymphocytes (Figure 4).

Role of D2-like receptors in mediating modulation of T lymphocyte function

Exposure of lymphocytes to D2-like receptor agonist quinpirole (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M) evoked a decrease in proliferative response of the lymphocytes to Con A stimulation (Figure 5A). This inhibitory effect of quinpirole was greater with its increasing concentrations, with a significant difference between the every two concentrations of quinpirole.

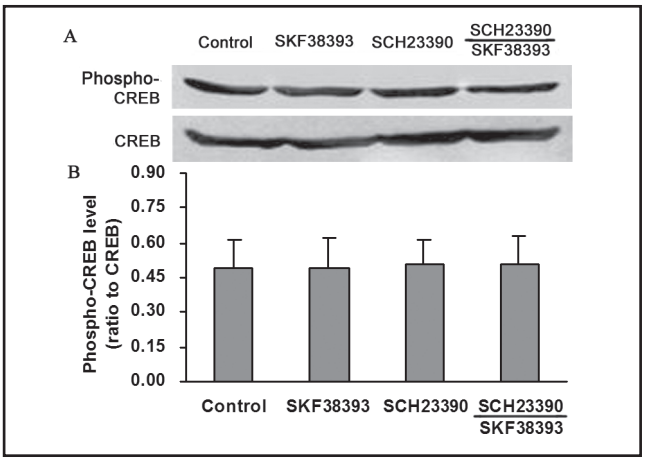

Fig. 4. Activation of lymphocyte D1-like receptors does not significantly influence CREB expression. The Con A-induced lymph node cells were exposed to dopamine D1-like receptor agonist SKF38393 (10^{-8} M) or antagonist SCH23390 (10^{-7} M) for 48 h. Nuclear protein was extracted and detected by Western blot with an antibody against phosphorylated CREB in Ser133. The same blots were then stripped and re-probed with a CREB antibody, which was used as an inner control. The data from three separate experiments did not exhibit statistic significance between the groups.

Combined treatment of lymphocytes with D2-like receptor antagonist haloperidol (10^{-10}, 10^{-9}, 10^{-8} or 10^{-7} M) and agonist quinpirole (10^{-8} or 10^{-7} M) caused the suppressed lymphocyte proliferation by the quinpirole to gradually recover to a control level without any drug, showing that haloperidol blocks the inhibitory effect of quinpirole on the Con A-induced lymphocyte proliferation. The haloperidol blockage depended on its concentration. At the lower concentration (10^{-10} M), haloperidol did not block the suppressed lymphocyte proliferation by the quinpirole; while at the higher concentration (10^{-7} M), haloperidol

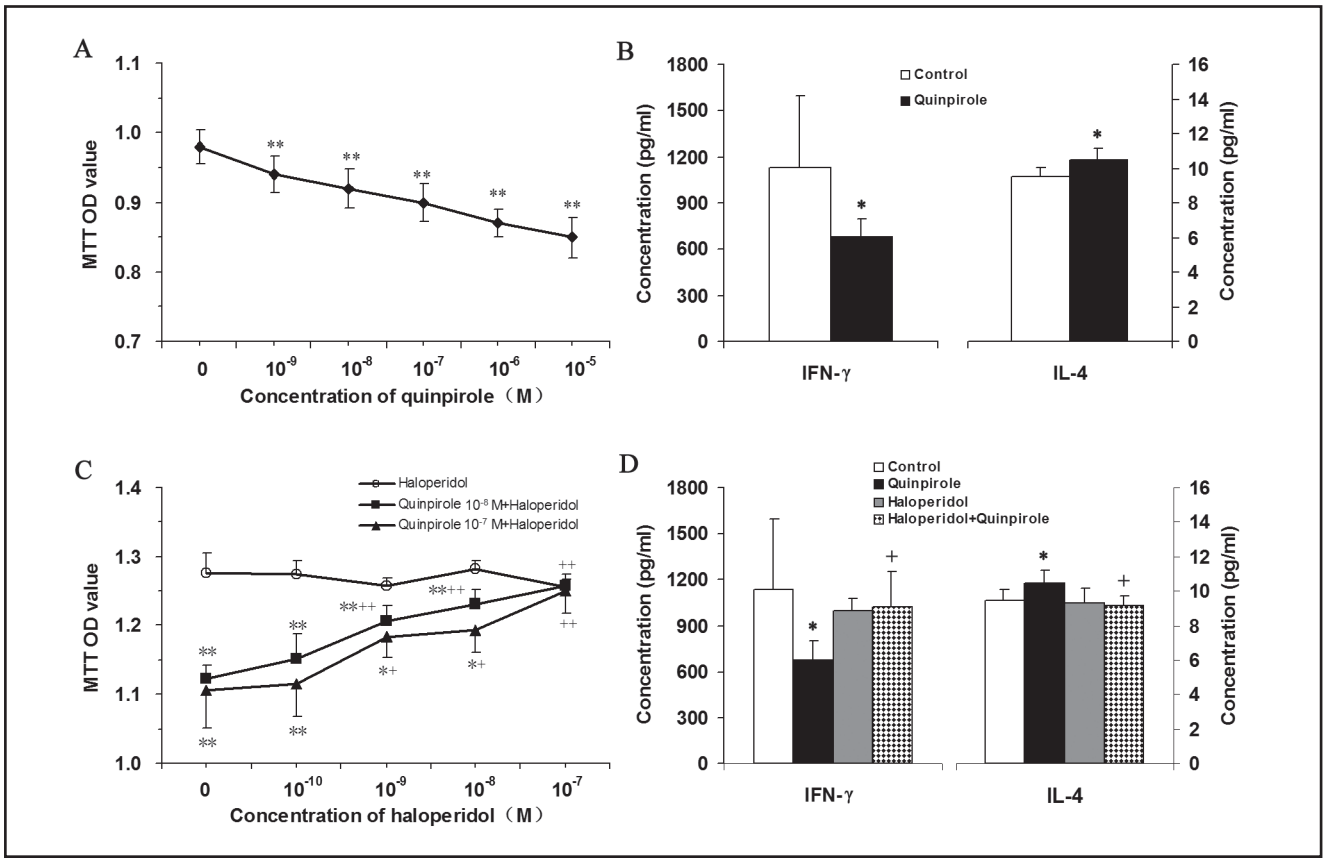

Fig. 5. Roles of lymphocyte D2-like receptors in mediating modulation of T cell function. The mesenteric lymph node cells were cultured with Con A for 48 h in the presence of D2-like receptor agonist quinpirole or antagonist haloperidol. MTT and cytometric bead array were used to measure the proliferative response of the lymphocytes as well as IFN-γ and IL-4 concentrations in the culture supernatants, respectively. (A) Influence of the D2-like receptor agonist quinpirole on the proliferative response of the lymphocytes to Con A. (B) Effects of quinpirole (10^{-8} M) on the production of the two cytokines, IFN-γ and IL-4, by the Con A-stimulation lymphocytes. (C) Blocking action of the D2-like receptor antagonist haloperidol on the quinpirole inhibition of Con A-induced lymphocyte proliferation. (D) Blocking effect of haloperidol (10^{-7} M) on the quinpirole-induced changes in IFN-γ and IL-4 production. The data were from eight (A) or five (B, C and D) separate experiments. *p<0.05, **p<0.01, compared with 0 point or control (i.e., without any drug treatment); +p<0.05, ++p<0.01, compared with quinpirole treatment alone.

completely reversed the attenuated lymphocyte proliferation by the quinpirole (Figure 5C). This blocking property of haloperidol was similar in its antagonizing the two different concentrations of quinpirole (10^{-7} and 10^{-8} M) (Figure 5C).

Besides, the quinpirole (10^{-8} M) exposure reduced IFN-γ, but elevated IL-4 levels in the supernatants of the Con A-stimulated lymphocyte cultures compared with the control (Figure 5B). The co-treatment with haloperidol (10^{-7} M) and quinpirole (10^{-8} M) caused the reduced IFN-γ and the elevated IL-4 levels by the quinpirole to return to the control levels (Figure 5D), indicating a blocking effect of haloperidol on the quinpirole-induced cytokine changes.

Effects of lymphocyte D2-like receptor activation on intracellular cAMP content and phosphorylated CREB expression

Intracellular cAMP content in the Con A-stimulated lymphocytes was decreased by the treatment with the D2-like receptor agonist quinpirole (10^{-8} or 10^{-7} M). The decreased cAMP content by quinpirole recovered almost to a control level lacking any drug by the co-treatment with the D2-like receptor antagonist haloperidol (10^{-8} or 10^{-7} M) and quinpirole (10^{-8} or 10^{-7} M) (Figure 6). This reversing effect of haloperidol on the quinpirole-decreased cAMP content occurred in any concentrations used (Figure 6).

Similarly, phosphorylated CREB expression in the Con A-stimulated lymphocytes was down-regulated by the quinpirole (10^{-8} M), and this effect was blocked by haloperidol (10^{-7} M) (Figure 7). The haloperidol treatment alone did not significantly influence the cAMP content and the phosphorylated CREB expression in the Con A-stimulated lymphocytes (Figures 6 and 7).

DISCUSSION

The purified T lymphocytes derived from the mesenteric lymph nodes of mice expressed all the five subtypes of dopamine receptor mRNAs, including D1R and D5R (D1-like receptors) and D2R, D3R and D4R (D2-like

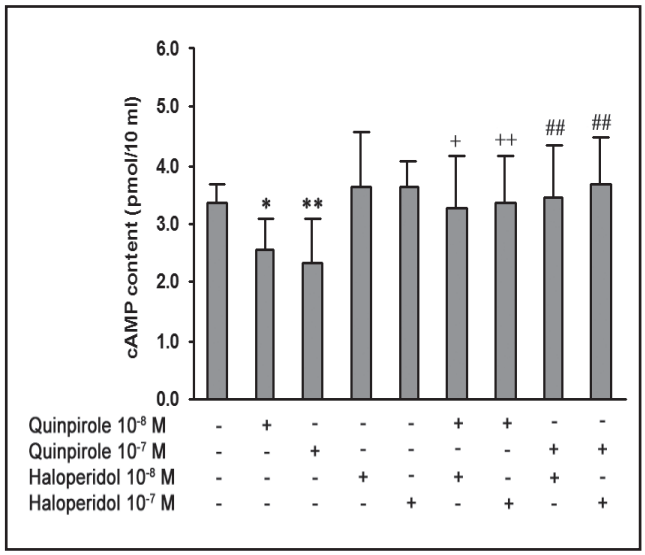

Fig. 6. Changes of cAMP content in the lymphocytes treated with quinpirole or with haloperidol plus quinpirole. The Con A-induced lymphocytes exposed to the D2-like receptor agonist quinpirole or to the antagonist haloperidol plus quinpirole for 48 h were measured by radioimmunoassay for their intracellular cAMP content. The data were obtained from eleven separate samples. *p<0.05, **p<0.01, compared with the control without any drug exposure; +p<0.05, ++p<0.01, compared with the 10^{-8} M of quinpirole group; ##p<0.01, compared with the 10^{-7} M of quinpirole group.

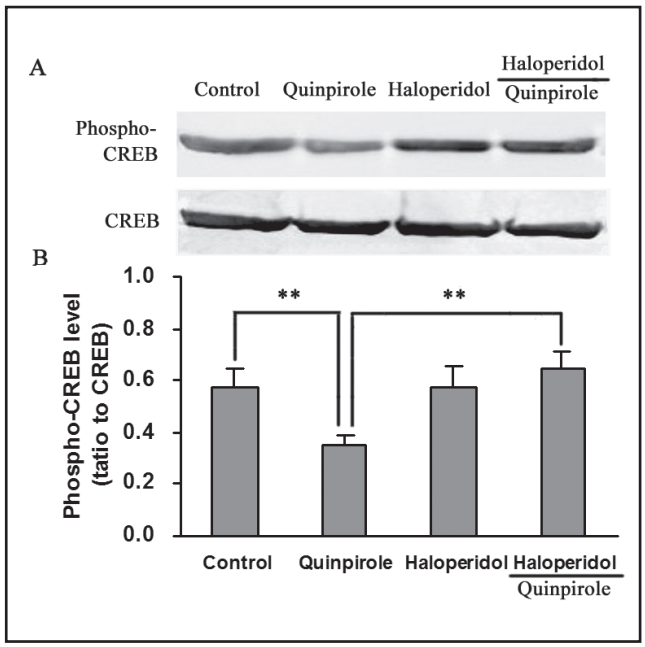

Fig. 7. Activation of lymphocyte D2-like receptors down-regulates the phosphorylated CREB expression. Lymph node cells were stimulated by Con A and exposed to quinpirole (10^{-8} M) or to haloperidol (10^{-7} M) and quinpirole for 48 h. Nuclear protein was extracted and detected by Western blot with an antibody against phosphorylated CREB in Ser133. The same blots were then stripped and re-probed with a CREB antibody. The data from three respective experiments were reported as phosphorylated CREB/CREB immunoreactivity. **p<0.01.

receptors). This suggests the presence of the five subtypes of dopamine receptors on murine T lymphocytes. At present, although some reports have pointed out the expression or presence of various subtypes of dopamine receptors in T lymphocytes, the reported results are not fully consistent, probably because of the different subpopulations of lymphocytes or the different research methods used in these studies. Especially, the facts that the dopamine D1R is expressed by rat lymphocytes (Caronti *et al.* 1998) but not by human lymphocytes (Ricci *et al.* 1999; Mckenna *et al.* 2002) propose a difference between murine and human lymphocytes. Our present data provide more evidence for the D1R expression in murine lymphocytes, and also establish a fact that all the five subtypes of dopamine receptors are expressed by T lymphocytes of mice. On the other hand, these facts also furnish a substantial basis for dopamine immunomodulation that is mediated by the subtypes of dopamine receptors.

The activation of lymphocyte D1-like receptors by SKF38393 neither changed the proliferative response of the lymphocytes to mitogen Con A, nor affected the production of IL-4 by the Con A-stimulated lymphocytes. However, IFN-γ production was decreased by the SKF38393 stimulation and this effect was blocked by D1-like receptor antagonist SCH23390. Since the two cytokines, IFN-γ and IL-4, are produced by different lymphocytes and play distinct roles in the immune system, their different responses to the D1-like receptor activation could be understood. IFN-γ is a pro-inflammatory cytokine produced mainly by T helper 1 (Th1) cells, and has a promoting effect on cellular immunity; while IL-4 is an anti-inflammatory cytokine produced mainly by Th2 cells, and has a facilitating action on humoral immunity (Abbas *et al.* 1996; Fearon & Locksley 1996; Mosmann & Sad 1996). Based on this, we suggest that dopamine D1-like receptors are related to the suppression of Th1 cell function and cellular immunity. In agreement with our observation, Carr *et al.* (2003) reported that the number of IFN-γ, but not IL-4-producing cells in spleen is significantly inhibited following treatment with L-dopa for 5 days, and this effect is replicated by infusion of dopamine and blocked by co-treatment with a dopamine antagonist, suggesting dopamine has a direct role in regulating immune responses through down-regulation of IFN-γ. However, a profound exploration of D1-like dopamine receptor-mediated immunomodulation is required to expand the comprehension of its functional significance.

The stimulation of lymphocyte D2-like receptors by quinpirole, a selective dopamine D2-like receptor agonist, diminished the proliferative response of the lymphocytes to Con A. This effect of quinpirole occurred in its wide concentration range and had an increasing action with its increasing concentration. Haloperidol, a D2-like receptor antagonist, blocked the inhibitory effect of quinpirole. The blockage of haloperidol depended on its concentration. At its lower

concentration (10^{-10} M), the haloperidol did not block the inhibition of the lymphocyte proliferation induced by quinpirole, but the higher concentration of haloperidol (10^{-7} M) completely reversed the inhibitory effect. These data strongly show that dopamine D2-like receptors are actively implicated in the modulation of T cell proliferation. Some reports have presented a regulation of T cell proliferation by dopamine. For example, dopamine can suppress T cell proliferation and cytotoxicity when it interacts with activated T cells (Levite 2008), and the inhibition of T cell proliferation by dopamine occurs at its physiological (Saha *et al.* 2001a, b) and pharmacological concentrations (Morikawa *et al.* 1994; Cook-Mills *et al.* 1995; Offen *et al.* 1995; Bergquist *et al.* 1998; Cioca *et al.* 2000). The consistent inhibition of T lymphocyte proliferation by the D2-like receptor activation and by the dopamine stimulation implies a receptor mechanism mediating the dopamine immunoinhibitory action, via D2-like receptors.

Furthermore, the Th1 cell-produced pro-inflammatory cytokine, IFN-γ, was reduced, but the Th2 cell-produced anti-inflammatory cytokine, IL-4, augmented by the D2-like receptor agonist quinpirole in this study. Both the effects of quinpirole were abolished by the D2-like receptor antagonist haloperidol. These results demonstrate that D2-like receptors are involved in the modulation of function of Th1 and Th2 cells. The opposite responses of the Th1 and Th2 cells to the D2-like receptor activation suggest a different effect of dopamine on immune function, i.e., suppressive on Th1 cell-mediated cellular immunity and enhancive on Th2 cell-mediated humoral immunity. This suggestion is supported by the reported evidence that dopamine inhibits lymphocyte proliferation, reduces the synthesis of pro-inflammatory and induces the synthesis of anti-inflammatory mediators (Beck *et al.* 2005). Interestingly, the dopamine D1-like and D2-like receptors had the same suppressive effects on the IFN-γ production, when they were activated. The phenomenon explains that dopamine has a marked and direct inhibitory action on Th1 cells and cellular immunity;. This explanation can be supported by the other studies (Cook-Mills *et al.* 1995; Tsao *et al.* 1997; Saha *et al.* 2001a, b; Carr *et al.* 2003; Beck *et al.* 2005). In the dopamine D2-like receptor subtypes, stimulation of D2R promotes production of IL-10, a cytokine that negatively regulates the function of effector T cells (45). D3R stimulation facilitates differentiation of naive CD8+ T cells into cytotoxic T lymphocytes (Besser *et al.* 2005), and it also contributes to polarization of naive CD4+ T cells toward Th1 effector phenotype (Ilani *et al.* 2004). Regarding D4R stimulation, evidence indicates that this receptor triggers T cell quiescence by up-regulating Krüppel-like factor-2 expression (Buckley *et al.* 2001; Sarkar *et al.* 2006). These findings, supported by our present results, confirm a potent participation of D2-like receptors in T lymphocyte regulation, and also reveal a diverse action mediated by these different dopamine receptor subtypes. On the basis of these findings, we suggest that D2-like receptors are more important in mediating regulation of T cell physiology than D1-like receptors.

In many cells, a classical signal-transduction pathway for dopamine D1-like receptors is cAMP second messenger cascade (Monsma *et al.* 1990; Sunahara *et al.* 1991; Jackson *et al.* 1994). In general, stimulation of D1-like receptors results in a generation of cAMP and subsequently activates PKA (Platzer *et al.* 2000), which regulates the activity of a number of transcription factors, such as CREB (Rohr *et al.* 1999; Vallone *et al.* 2000; Beck *et al.* 2004). In the present study, the stimulation of lymphocyte D1-like receptors by the receptor agonist SKF38393 did not significantly affect the cAMP content and the CREB activation in the cells. It implies that in lymphocytes, the classical cAMP second messenger cascade may be not a prevailing signal-transduction pathway for the D1-like receptors. Thereby, we suggest that the SKF38393-induced decrease in IFN-γ is not mediated by the cAMP-PKA linked CREB pathway. The suggestion is supported by the report from Ghosh *et al.* (2003). They found that this action of dopamine inhibiting cytokine release through D1-like receptors is mediated by a new mechanism, i.e., dopamine suppresses non-receptor tyrosine kinases, Lck and Fyn expression which are the initial and pivotal signaling steps in T cell receptor mediated different down stream signaling cascades, leading to cytokine release and subsequent clonal expansion of these immune effector cells. In addition, the signal-transduction pathways activated by D1-like receptors are numerous (Vallone *et al.* 2000). For example, the D1-like receptor agonist SKF38393 increases the levels of p44/42 mitogen-activated protein kinases (MAPKs), p38 MAPK, and stress-activated protein kinase/Jun-N-terminal kinase (SAPK/JNK) phosphorylation (Lee *et al.* 2006); in vascular smooth muscle cell, the inhibited migration, proliferation and hypertrophy by D1-like receptor agonists are implemented possibly through the suppression of activated PLD, PKC and MAPK activity (Yasunari *et al.* 1999). Therefore, the reduced IFN-γ by the D1-like receptor stimulation in this study is possible to be mediated by other signaling rather than the cAMP/PKA linked CREB pathway. Nevertheless, this remains to be verified.

Dopamine D2-like receptors have been known to be negatively coupled to cAMP/PKA-dependent signaling (Vallone *et al.* 2000; Yao *et al.* 2008). Our current findings indicated that the cAMP content and the phosphorylated CREB expression in the Con A-activated lymphocytes were depressed by the D2-like receptor agonist quinpirole, and that the receptor antagonist haloperidol blocked these effects of quinpirole. The evidence demonstrates that activation of lymphocyte D2-like receptors suppresses the downstream signal pathway, cAMP/PKA-CREB pathway, and therefore testifies a negative association of D2-like receptors with cAMP/PKA-CREB signaling in lymphocytes. Thus, these changes in T cell proliferation and cytokine pro-

duction in this study can be considered as an outcome of cAMP/PKA-CREB signal-transduction inhibition induced by dopamine D2-like receptor activation in the lymphocytes.

In summary, T lymphocytes expressed all the five subtypes of dopamine receptors, including D1R and D5R (D1-like receptor family) and D2R, D3R, and D4R (D2-like receptor family). Activation of the D1-like receptors only reduced the IFN-γ production by the Con A-stimulated lymphocytes; while activation of the D2-like receptors inhibited the proliferative response of lymphocytes to Con A, and decreased the IFN-γ but elevated the IL-4 levels. These results imply a different effect of dopamine on Th1 and Th2 cell function, inhibitory on Th1 cells and enhancive on Th2 cells, and also propose a predominant role of D2-like receptors in mediating the modulation of T cell function. In addition, the activation of the D2-like receptors diminished the intracellular cAMP content and down-regulated the phosphorylated CREB expression in the Con A-stimulated lymphocytes. This suggests that the modulation of T cell function by dopamine is implemented by cAMP-linked CREB pathway negatively coupled to D2-like receptors.

ACKNOWLEDGEMENTS

This work was supported by grants 2007-5 for Excellent Sci-Tech Innovation Teams in Universities from the Educational Department of Jiangsu Province of China, H200761 from the Health Department of Jiangsu Province of China, 30870819 and 30870929 from the National Natural Science Foundation of China, and K2008006 and K2008019 from the Nantong Applied Research Program of China.

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