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Acetylcholine polarizes dendritic cells toward a Th2-promoting profile

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Keywords
acetylcholine; cholinergic system; dendritic cells; thymic stromal lymphopoietin.

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Abstract

Background: A growing body of research shows a reciprocal regulation between the neural and immune systems. Acetylcholine (ACh) is the most important parasympathetic neurotransmitter, and increasing evidence indicates that it is able to modulate the immune response. Interestingly, in recent years, it has become clear that immune cells express a non-neuronal cholinergic system, which is stimulated in the course of inflammatory processes. We have previously shown that dendritic cells (DC) express muscarinic receptors, as well as the enzymes responsible for the synthesis and degradation of ACh. Here, we analyzed whether ACh could also modulate the functional profile of DC.
Methods: Dendritic cells were obtained from monocytes cultured for 5 days with GM-CSF+IL-4 or isolated from peripheral blood (CD1c+ DC). The phenotype of DC was evaluated by flow cytometry, the production of cytokines was analyzed by ELISA or intracellular staining and flow cytometry, and the expression of muscarinic and nicotinic receptors was evaluated by flow cytometry or qRT-PCR.
Results: Treatment of DC with ACh stimulated the expression of the Th2-promoter OX40L, the production of the Th2-chemokines MDC (macrophage-derived chemokine/CCL22) and TARC (thymus and activation-regulated chemokine/CCL17), and the synthesis of IL-4, IL-5, and IL-13 by T cells, in the course of the mixed lymphocyte reaction (MLR). Moreover, we found that the stimulation of OX40L, HLA-DR, and CD83 expressions in DC induced by the Th2-promoting cytokine TSLP, as well as the production of IL-13, IL-4, and IL-5 by T cells in the course of the MLR, was further enhanced when DC were treated with TSLP plus ACh, instead of TSLP or ACh alone.
Conclusions: Our observations suggest that ACh polarizes DC toward a Th2-promoting profile.

Abbreviations
ACh, acetylcholine; AT, atropine; DC, conventional dendritic cells; M, muscarinic receptors; MDC, macrophage-derived chemokine; MLR, mixed lymphocyte reaction; MM, mecamylamine; nAChR, nicotinic receptors; PAMPs, pathogen-associated molecular patterns; PBMC, peripheral blood mononuclear cells; TARC, thymus and activation-regulated chemokine; TSLP, thymic stromal lymphopoietin; TSLPR, TSLP receptor.

Upon encountering pathogens or inflammatory cytokines in peripheral tissues, conventional dendritic cells (DC) undergo a set of important changes leading to their maturation in antigen-presenting cells, competent to induce both, the activation of resting T cells and their differentiation into distinct functional profiles (1,2). However, besides pathogen-associated molecular patterns (PAMPs) and cytokines, other stimuli are known to be able to modulate the function of DC (3).

Lymphoid tissues are innervated by the autonomic nervous system (4). Moreover, a large body of evidence supports the existence of a non-neuronal cholinergic system in immune cells, which is activated during the course of inflammatory processes (4–7). In fact, the components of the cholinergic system, acetylcholine (ACh), muscarinic and nicotinic receptors, acetylcholinesterase, and choline acetyltransferase are expressed by B and T cells, and ACh has been shown to modulate the function of macrophages and T $CD8^+$ cells (4,8,9). We have recently reported that DC express muscarinic receptors, as well as the enzymes responsible for the production and degradation of ACh. Moreover, we found that ACh modulates the function of DC. Of note, ACh induced opposite effects depending on the activation status of DC; it increased the expression of HLA-DR and CD86 and the production of TNF-α by resting DC, but significantly inhibited the stimulation of HLA-DR and CD86 expressions, and the production of TNF-α in LPS-activated DC (10).

Acetylcholine is the most important parasympathetic neurotransmitter in the airways (11). It induces airway smooth muscle contraction, and for this reason, anticholinergics are usually used as bronchodilators in obstructive airway diseases (11–13). Interestingly, recent studies reveal that ACh not only induces bronchoconstriction but also promotes changes in the airways associated to Th2 responses such as eosinophilia, mucus overproduction, and airway remodeling (11,14,15).

In this study, we analyze whether ACh could induce the polarization of DC toward a Th2-promoting profile. Moreover, considering the important role played by thymic stromal lymphopoietin (TSLP) in the development of allergic processes (16,17), we also analyzed whether ACh could modulate the ability of TSLP to promote a Th2 response.

Materials and methods

The studies performed in this work have been reviewed and approved by the Research Ethics Committee of the 'Academia Nacional de Medicina', CABA, Argentina. All blood donors provided a written informed consent for the collection of samples and subsequent analyses.

Reagents

Endotoxin-free reagents and plastic materials were used in all experiments. RPMI-1640, fetal calf serum (FCS), penicillin/streptomycin, and phosphate-buffered saline (PBS) 10× were purchased from Gibco, Life Technologies (Waltham, MA, USA); 96-well U-bottom, 6-well polystyrene plates and half-area 96-well ELISA plates from Greiner Bio One, GBO (Frickenhausen, Germany); recombinant human IL-4, recombinant human granulocyte/macrophage colony-stimulating factor (GM-CSF) from Miltenyi Biotec (Bergisch Gladbach, Germany); ACh, carbamylcholine chloride (carbachol), and mecamylamine (MM) from Sigma-Aldrich (St. Louis, MO, USA); atropine (AT) from Laboratorio Larjan (Buenos Aires, Argentina); TSLP from R&D Systems (Minneapolis, MN, USA); IL-12 from PeproTech (Rocky Hill, CT, USA) and FIX and PERM kit from Dako, Agilent Technologies (Glostrup, Denmark).

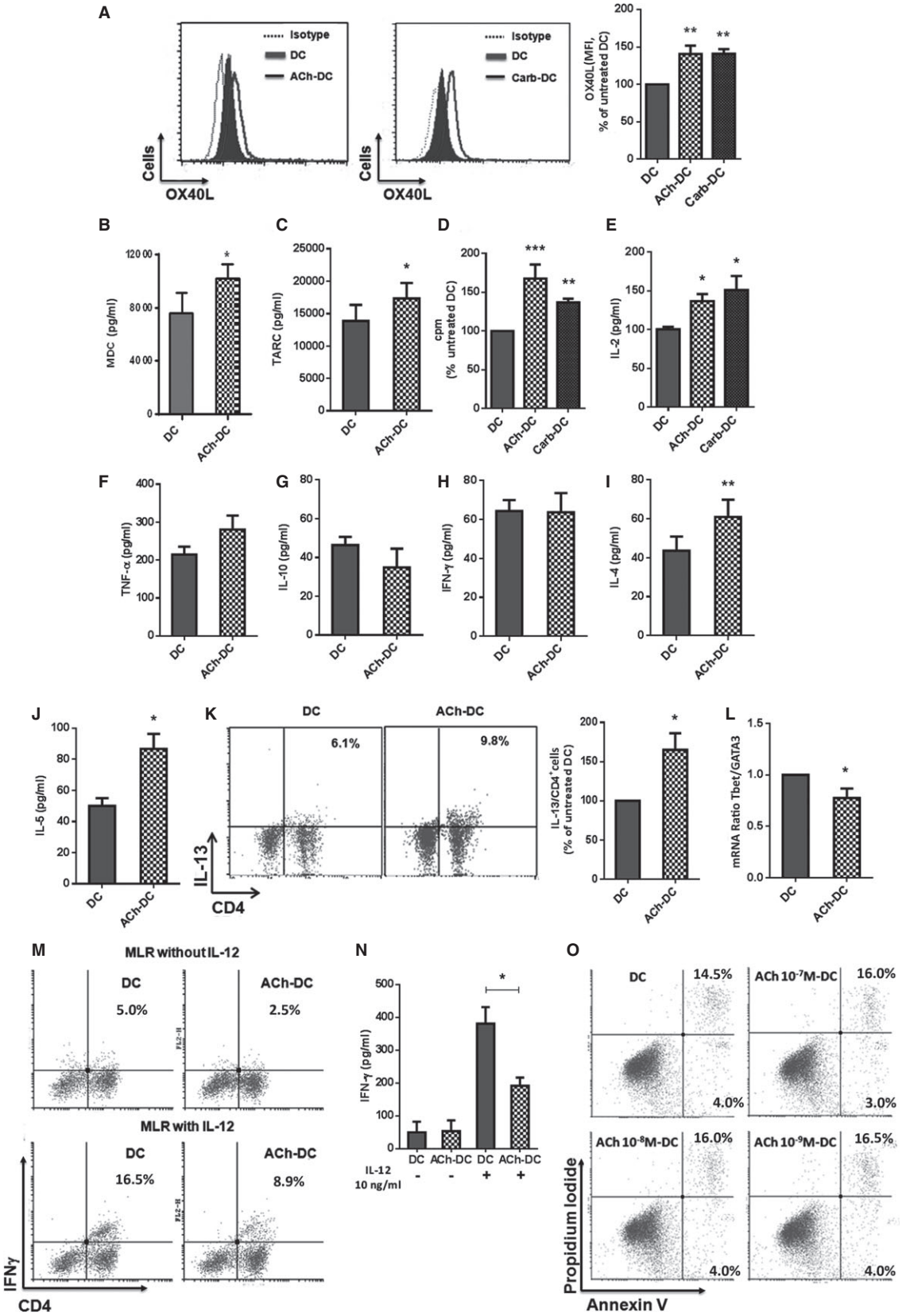
Preparation of DC

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy adult nonsmoker volunteer blood donors from the 'Servicio de Medicina Transfusional, Hospital Italiano' (Buenos Aires, Argentina) by Ficoll-Hypaque (GE Healthcare Life Sciences, Freiburg, Germany) density gradient centrifugation. $CD14^+$ cells were then isolated by positive selection according to the manufacturer's instructions (Miltenyi Biotec). Purity was assessed by FACS analysis using anti-CD14 mAb and was found to be >95%. To obtain DC, monocytes (1×10^6/ml) were cultured in complete medium (RPMI 1640 medium supplemented with 10% of heat-inactivated FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin) with 20 ng/ml IL-4 and 20 ng/ml GM-CSF, in 6-well culture plates. On day 5, cells were analyzed by FACS, and the expression of CD1a, as a marker of DC differentiation, was evaluated.

Isolation of CD1c (BDCA-1)$^+$ myeloid DC from peripheral blood

CD1c $(BDCA\text{-}1)^+$ myeloid DC (CD1c+ DC) were isolated from peripheral blood of healthy adult nonsmoker volunteers

Figure 1 Acetylcholine polarizes dendritic cells toward a Th2-promoting profile. (A-C) DC (1×10^6/ml) were cultured for 18 h with or without ACh or carbachol (10^{-8}M) and the expression of OX40L was evaluated by flow cytometry (A), while the production of MDC and TARC was measured by ELISA (B and C). In (A), representative histograms are shown in the left and center panels, while the mean ± SEM of the mean fluorescence intensity or MFI ($n = 8$) is shown in the right panel. (D-L) DC (1×10^6/ml) were cultured for 18 h with or without ACh or carbachol (10^{-8}M), washed and cultured with allogeneic lymphocytes (DC/lymphocyte ratio 1 : 5). Proliferation of lymphocytes (D); production of the cytokines IL-2 (E) TNF-α (F), IL-10 (G), IFN-γ (H), IL-4 (I), IL-5 (J) (ELISA), IL-13 (intracellular staining and flow cytometry) (K); and the mRNA expression ratio of Tbet/Gata3 (L) were determined. Levels of IL-2 were evaluated at day 2 of culture, while the other assays were made at day 5. Results represent the mean ± SEM of 5-8 experiments. Panel K, left, a representative experiment is shown. In panel (L), a relative value of 1 was given to the mRNA expression ratio of Tbet/Gata3 for cultures performed with untreated DC. (M-N) DC (1×10^6/ml) were cultured for 18 h with or without ACh (10^{-8}M), washed and cultured with allogeneic lymphocytes (DC/lymphocyte ratio 1 : 5), in the absence or presence of IL-12 (10 ng/ml). After 5 days of culture, the production of IFN-γ was evaluated by intracellular staining and flow cytometry (M) or ELISA (N). A representative experiment is shown in M, and the mean ± SEM of 4 experiments is shown in N. (O) Apoptosis and viability were evaluated using an Annexin V-FITC/propidium iodide kit and flow cytometry. A representative experiment ($n = 3$) is shown. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* controls (DC).



by positive selection of CD1c+ (Miltenyi, % purity >95) according to the manufacturer's instructions.

Purification of naïve CD4+ T cells

Human naïve $CD4^+$ T cells were isolated from PBMC by depletion of cells expressing CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCR γδ, HLA-DR, and CD235a (glycophorin A), using magnetic beads according to the manufacturer's instructions (Miltenyi, >95% purity). $CD4^+$ T cells (1×10^6 cells/ml) were suspended in RPMI 1640 medium supplemented with 10% of heat-inactivated human AB Serum, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Mixed Lymphocyte Reaction (MLR)

Dendritic cells were cultured for 18 h in the absence or presence of ACh/Carbachol and/or TSLP. Then, cells were washed and cultured alone or in the presence of freshly isolated allogeneic lymphocytes (DC/lymphocyte ratio = 1/5) for 5 or 7 days in 96-well U-bottom plates. Thymidine incorporation was measured at day 5 by a 16 h pulse with [^{3}H] thymidine (1 μCi/well, specific activity, 5 Ci/mM; DuPont, Wilmington, DE, USA). The production of cytokines was evaluated in cell supernatants by ELISA or by intracellular staining and flow cytometry, in cells previously treated for 4 h with brefeldin A (10 μg/ml, GolgiPlug, BD Biosciences, San Diego, CA, USA).

Flow cytometry

Cells were washed twice with PBS supplemented with 2% FCS and resuspended in PBS supplemented with 10% heat-inactivated FCS. FITC- and PE-conjugated mAbs were added at saturating concentrations for 30 min at 4°C, and two additional washes were then performed. FITC- or PE-conjugated mAbs directed to CD1a, CD14, CD19, CD4, CD40, CD86, HLA-DR, CD83 (BD Biosciences), OX40L (BioLegend, San Diego, CA, USA), CD1c, and CD45RA (Miltenyi) were used. Analysis was performed using a FACS flow cytometer and CellQuest software (BD Biosciences). Production of IL-13 was analyzed by intracellular staining and flow cytometry using a PE-conjugated mAb direct to IL-13 (BioLegend). The expression of muscarinic receptors was evaluated using specific goat or rabbit IgG polyclonal antibodies directed to the muscarinic receptors M1, M2, M3, M4, or M5 (Santa Cruz, Biotechnology, Heidelberg, Germany) and secondary FITC-labeled polyclonal IgG antibodies directed to goat or rabbit IgG (Sigma-Aldrich). Analysis was performed using a FACS flow cytometer and CellQuest software (BD Biosciences).

Quantitation of DC Apoptosis by Annexin V Binding and Flow Cytometry

Annexin V binding to DC was performed using an apoptosis detection kit (Sigma-Aldrich).

In brief, cells were labeled with annexin V-FITC for 20 min at 4°C and with propidium iodide immediately before evaluation of fluorescence by flow cytometry. Results are expressed as percentage of annexin V-positive cells.

Measurement of cytokines by ELISA

Cytokines were evaluated in cell supernatants using commercial kits: TNF-α (E-Biosciences, San Diego, CA, USA); IL-2, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-17, IFN-γ (BD Biosciences); MDC (macrophage-derived chemokine/CCL22); TARC (thymus and activation-regulated chemokine/CCL17) (R&D Systems); and IL-4 (Immunotools, Friesoythe, Germany), according to the manufacturer's recommendations.

Quantitative real-time RT-PCR

Total RNA from DC was obtained from 1×10^6 cells or MLR after 5 days, using TRIzol reagent (Invitrogen, Life Technologies, CA, USA) according to the manufacturer's instructions. The reverse transcription reactions were carried out using the Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA) following the manufacturer's procedure. Quantitative real-time PCR (qRT-PCR) was performed using an ABI 7500 cycler (Applied Biosystems, Foster City, CA, USA) with the SYBR Green PCR Select Master Mix (Applied Biosystems), according to the protocol of the manufacturer. Primer sequences used were as follows: *GAPDH* forward: 5′-GAGTCAACGGATTTGGTCGT-3′, reverse: 5′-TTGATTTTGGAGGGATCTCG-3′; *TBET* forward: 5′-TACCCGGGGCCGCGTGAGGACTA-3′, reverse: 5′-CGGGTG GACGTACAGGCGGTTTC-3′; *GATA3* forward: 5′-TGTGTGAACTGTGGGGCAACCTCG-3′, reverse: 5′-TTTTCGGTTTCTGGTCTGGATGCC-3′; and α7 nicotinic receptor subunit gene (*CHRNA7*) forward: 5′-AGAACCAAGTTTTAACCACCAACA-3′, reverse: 5′-TAGTGTGGAATGTGGCGTCAA-3′. The specific primers for TSLP receptor gene (*TSLPR*) were from QuantiTect Primer Assay QT01156176 (Qiagen, Cambridge, MA, USA). *TBET*, *GATA3*, and *CHRNA7* primers were designed with Primer-BLAST online software. All qRT-PCR assays were performed in duplicate. *TSLPR*, *CHRNA7*, *TBET,* and *GATA3* expression was quantified relative to the mRNA expression of the endogenous reference gene *GAPDH* by comparative Ct method, using the $2^{-\Delta\Delta Ct}$ calculation. cDNA obtained from total RNA of DC treated with LPS (Sigma-Aldrich, 100 ng/ml) for 18 h was used as positive control of *TSLPR* expression (18), and the ratio of *TBET*/*GATA3* gene expression was calculated to evaluate the Th1/Th2 balance.

Statistical analysis

GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) was used to perform all statistical tests. Statistical significance was determined using the nonparametric Kruskal–Wallis test for multiple comparisons with Dunn's post-test analysis or Student's *t*-test. Statistical significance was defined as $P < 0.05$.

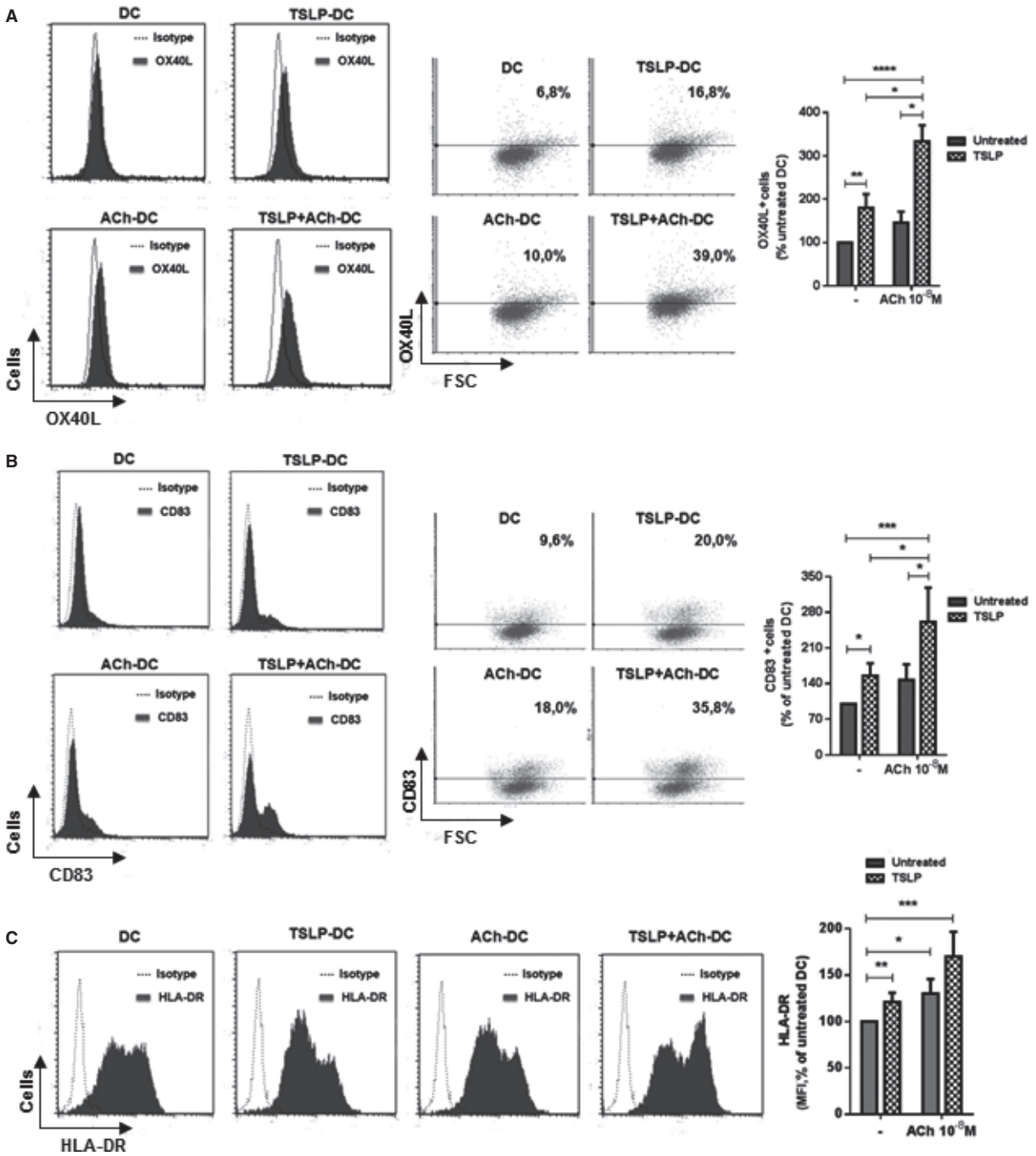


Figure 2 ACh increases the upregulation of OX40L, CD83, and HLA-DR in TSLP-stimulated DC. DC (1×10^6/ml) were cultured in the presence of ACh (10^{-8}M) and/or TSLP (15 ng/ml) for 18 h. Then, the expression of OX40L (A), CD83 (B), and HLA-DR (C) was evaluated by flow cytometry. Representative experiments or the mean ± SEM of 5-8 experiments are shown. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Results

Acetylcholine polarizes dendritic cells toward a Th2-promoting profile

Considering the important role played by the OX40–OX40L interaction in the induction of allergic inflammation (16,18), we analyzed whether ACh and the ACh analogue carbachol could be able to stimulate the expression of OX40L by DC. Based on our previous results (10), this first set of experiments was performed using 10^{-8} M of ACh or carbachol. Figure 1A shows that the culture of DC with either ACh (ACh-DC) or carbachol (Carb-DC) resulted in an increased

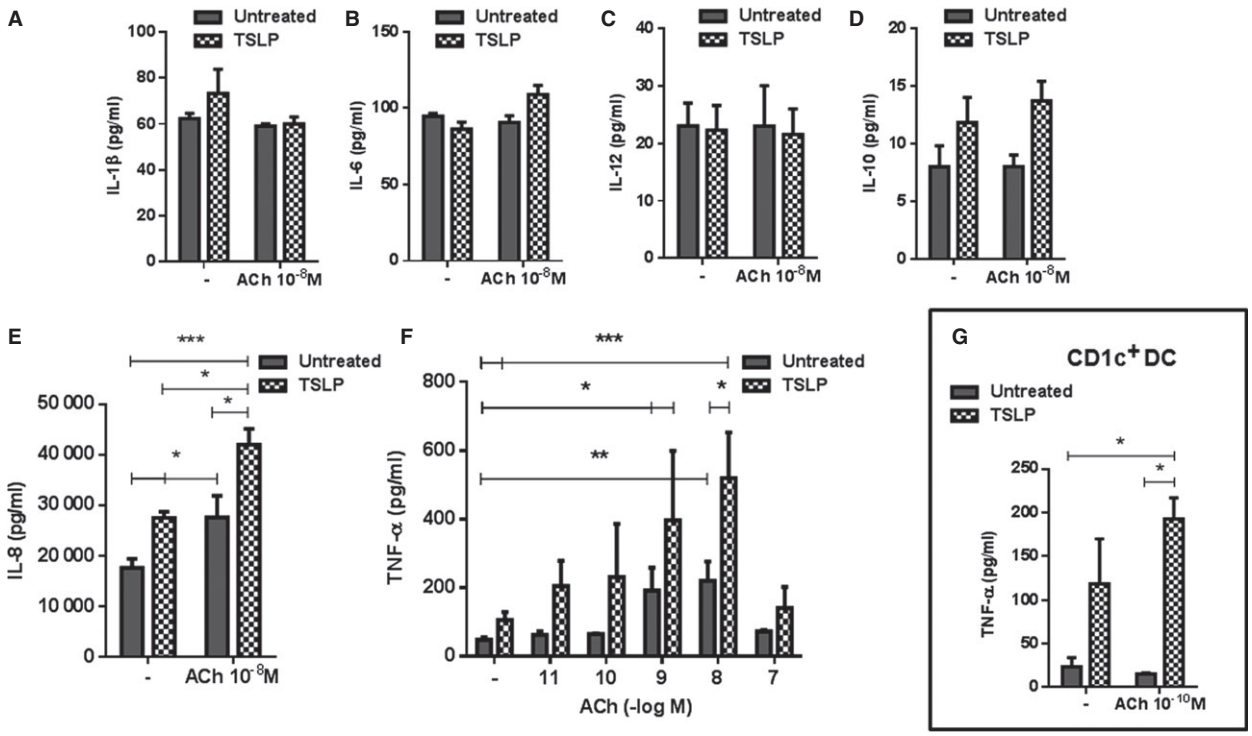


Figure 3 ACh increases the production of TNF-α and IL-8 in TSLP-stimulated DC. (A-G) DC (1×10^6/ml) were cultured in the presence of ACh and/or TSLP (15 ng/ml) for 18 h. Then, the production of cytokines was evaluated by ELISA. Results represent the mean ± SEM of 5-7 experiments. Panel (G) illustrates experiments performed with CD1c+ DC isolated from peripheral blood, instead of DC obtained from monocytes cultured with IL-4 and GM-CSF for 5 days. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

expression of OX40L. Moreover, incubation of DC with ACh also resulted in the stimulation of the production of MDC (macrophage-derived chemokine/CCL22) and TARC (thymus and activation-regulated chemokine/CCL17), two chemokines able to recruit Th2 cells at allergic inflammation sites (16,19) (Fig. 1B,C). Further experiments were performed to analyze the function of ACh-DC in the MLR. Treatment of DC with ACh or carbachol resulted in enhanced proliferation of allogeneic lymphocytes and stimulation of IL-2 production (Fig. 1D,E). No differences were observed in the production of cytokines TNF-α, IL-10, and IFN-γ (Fig. 1F-H), while the production of IL-17A was undetectable (not shown). Interestingly, ACh significantly increased the production of IL-4, IL-5, and IL-13 in the MLR (Fig. 1 I–K). Consistent with these observations, we also found that the ratio between the transcription factors Tbet/GATA3 was significantly lower in the MLR performed with ACh-DC compared with untreated DC (Fig. 1L*)*. Together, these observations suggest that ACh favors the polarization of DC toward a Th2-promoting phenotype. This was further confirmed when the function of ACh-DC was analyzed in the MLR performed under Th1-promoting conditions, that is in the presence of IL-12. Treatment of DC with ACh resulted in a lower production of IFN-γ compared with untreated DC (Fig. 1M,N). Finally, and to rule out that differences between ACh-DC and untreated DC could be related to changes in cell viability, we analyzed whether ACh could modulate the survival of DC. Figure 1O shows no differences between the viability of untreated and ACh-DC.

Acetylcholine favors the polarization of dendritic cells toward a Th2-promoting profile induced by TSLP

Because TSLP effectively induces the polarization of DC toward a Th2-promoting profile, we next analyzed whether ACh could be able to modulate this response. Figure 2A shows that incubation of DC with both TSLP and ACh resulted in the expression of higher levels of OX40L compared with those induced by each stimulus acting alone. A similar response was observed when the expression of CD83 and HLA-DR was analyzed (Fig. 2B,C). We then studied the production of cytokines by DC cultured with TSLP and/or ACh. Figures 3A-D show that neither TSLP nor ACh stimulated the production of IL-1β, IL-6, IL-12p70, and IL-10 by DC. By contrast, either TSLP or ACh stimulated the production of IL-8 and TNF-α with the production of both cytokines being higher for DC stimulated with TSLP plus ACh compared with those cells incubated with each stimulus alone (Fig. 3E,F). Similar results were observed when we analyzed the production of TNF-α by DC isolated from PBMC (CD1c+ DC) instead of DC obtained *in vitro* by differentiation of monocytes in the presence of IL-4 plus GM-CSF. Figure 3G shows that ACh increased the production of TNF-α triggered by TSLP in circulating myeloid CD1c+ DC.

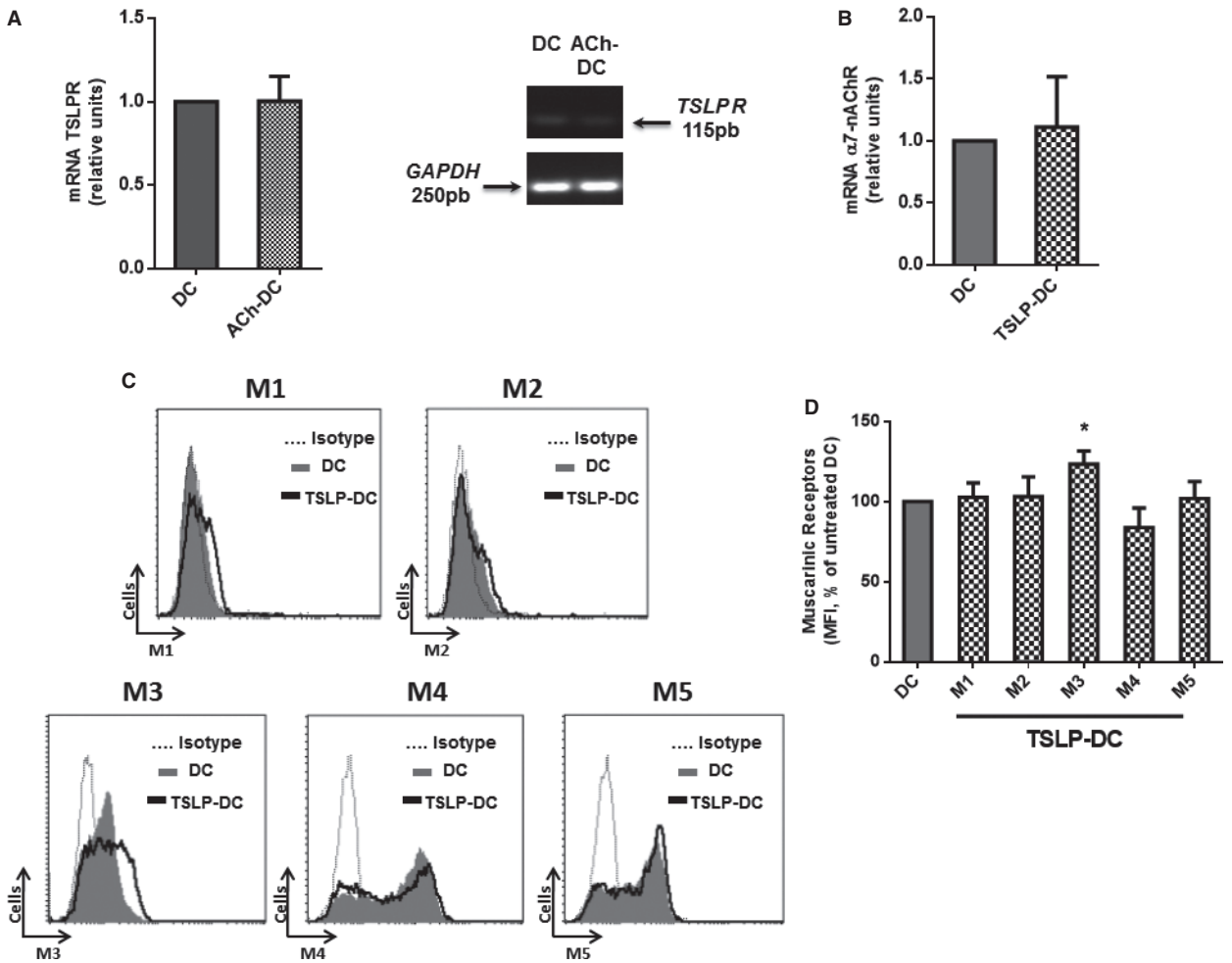


Figure 4 Effect of ACh and TSLP on the expression of TSLPR and cholinergic receptors by DC. DC (1×10^6/ml) were cultured with or without ACh (10^{-8}M) or TSLP (15 ng/ml) for 18 h and the expression of TSLPR (A), the α7 nicotinic receptor for ACh (α7-nAChR) (B), or muscarinic receptors (M1-M5) (C-D) was evaluated by qRT-PCR or flow cytometry, respectively. A relative value of 1 was given to the expression of mRNA for TSLPR and α7-nAChR for untreated DC. The mean ± SEM of 5 experiments is shown in A and B. Histograms of representative experiments performed by flow cytometry are shown in C, and the increase in the fluorescence intensity (MFI) over the controls (mean ± SEM) of 5 experiments is shown in D. $*P < 0.05$ *vs* controls (DC).

We then analyzed whether the enhancing effect induced by ACh on TSLP-mediated responses could be related to changes in the expression of TSLPR. The TSLPR is a heterodimeric protein composed of the TSLP receptor chain and the IL-7Rα chain (20,21). We analyzed the presence of TSLPR by qRT-PCR by measuring the expression of the mRNA for TSLPR chain. Figure 4A shows that ACh did not modify the expression of the TSLPR chain in DC. We also found that TSLP did not change the expression of the nicotinic receptor α7 (Fig. 4B) but significantly enhanced the expression of the muscarinic receptor M3 without modifying the expression of the receptors M1, M2, M4, and M5 (Fig. 4C and D).

Because DC express both muscarinic and nicotinic receptors for ACh, we then analyzed which of these receptors was involved in the promotion of TSLP-mediated effects induced by ACh. To this aim, we used nonselective muscarinic and nicotinic receptor antagonists: AT and MM, respectively. In these experiments, DC were incubated for 30 min with or without the cholinergic receptor antagonists, and then cells were cultured for 18 h with ACh, TSLP, or ACh plus TSLP. Figures 5A-D show that AT, but not MM, significantly inhibited the enhancing effect induced by ACh on the ability of TSLP to increase OX40L and CD83 expressions, as well as the production of TNF-α and IL-8, suggesting the involvement of muscarinic receptors.

Further experiments were then performed to compare the function of ACh-, TSLP- and TSLP+ACh-treated DC in the MLR. As shown in Figures 6A-F, the production of IL-2, TNF-α, IL-5, IL-4, and IL-13 induced in the course of MLR

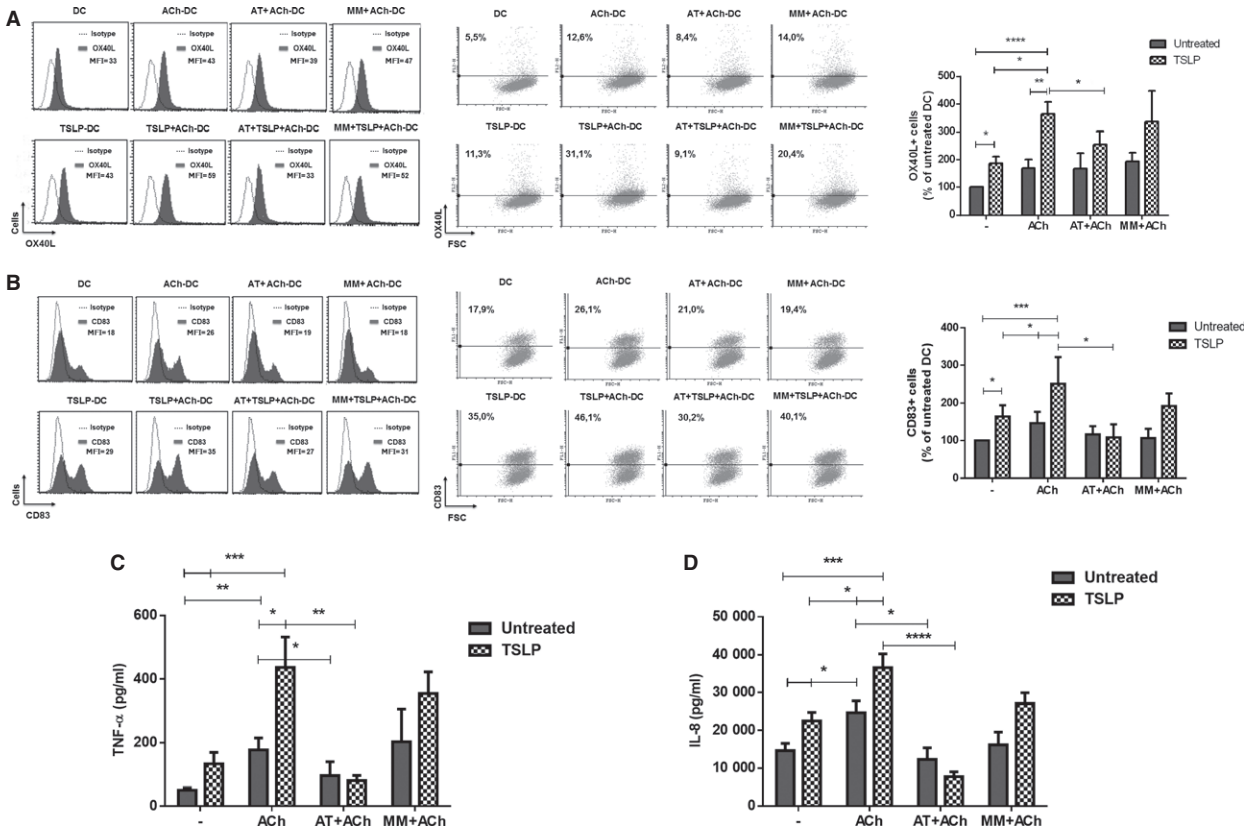


Figure 5 Atropine prevents the ability of ACh to increase the upregulation of OX40L and CD83 and the production of TNF-α and IL-8 in TSLP-stimulated DC. DC (1×10^6/ml) were pretreated for 30 min with or without the muscarinic receptor antagonist AT (10^{-7}M) or the nicotinic receptor antagonist MM (10^{-7}M). Then, cells were cultured in the presence of ACh (10^{-8}M) and/or TSLP (15 ng/ml) for 18 h, and the expression of OX40L (A) and CD83 (B) was evaluated by flow cytometry, while the production of TNF-α (C) and IL-8 (D) was assessed by ELISA. (A and B): Representative experiments are shown in left and central panels. The mean ± SEM of 4-5 experiments is shown in the right panel. (C and D) Results are expressed as the mean ± SEM of 5-8 experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

was significantly higher in cultures performed with TSLP+ACh-treated DC compared with those performed with DC treated with ACh or TSLP alone. Similar results were observed when the production of IL-2, TNF-α, and IL-5 was analyzed in the MLR induced by DC isolated from PBMC (CD1c+ DC) (Fig. 6G-I).

Discussion

Our results suggest that ACh polarizes DC toward a Th2-promoting profile. OX40L is a costimulatory molecule that stimulates the differentiation of $CD4^+$ T cells into a Th2 profile (18,22). ACh not only stimulated the expression of OX40L by DC but also increased the production of MDC and TARC, two chemokines that guide the recruitment of Th2 cells to allergic inflammation sites (16,19). Our results are consistent with those previously described by Liu et al. (23). They reported that the nonselective muscarinic receptor agonist methacholine increased the expression of OX40L by DC isolated from surgically removed nasal mucosa. Together, these results suggest that the ability of cholinergic system to stimulate the expression of OX40L is not restricted to a particular DC type.

Because TSLP has been shown to play a critical role in the induction and maintenance of allergic responses at epithelial surfaces, we also analyzed whether the actions mediated by TSLP on DC could be facilitated by ACh. As reported previously (18,24), we observed that TSLP increased OX40L expression by DC. Interestingly, we found that DC cultured with ACh plus TSLP showed levels of OX40L expression higher than those cells cultured with each stimulus alone. This suggests that ACh could further promote the induction of a Th2 profile, even in the presence of a strong Th2 inducer, such as TSLP. A similar effect was observed when the expression of HLA-DR and CD83 was analyzed. The expression of these maturation markers was higher in DC cultured with ACh plus TSLP compared with those cells cultured with each stimulus alone, suggesting that ACh could also facilitate the process of DC maturation induced by TSLP. Atropine, but not mecamylamine, significantly prevented the enhancing effect induced by ACh on the ability of TSLP to increase OX40L and CD83 expressions, as well as the production of

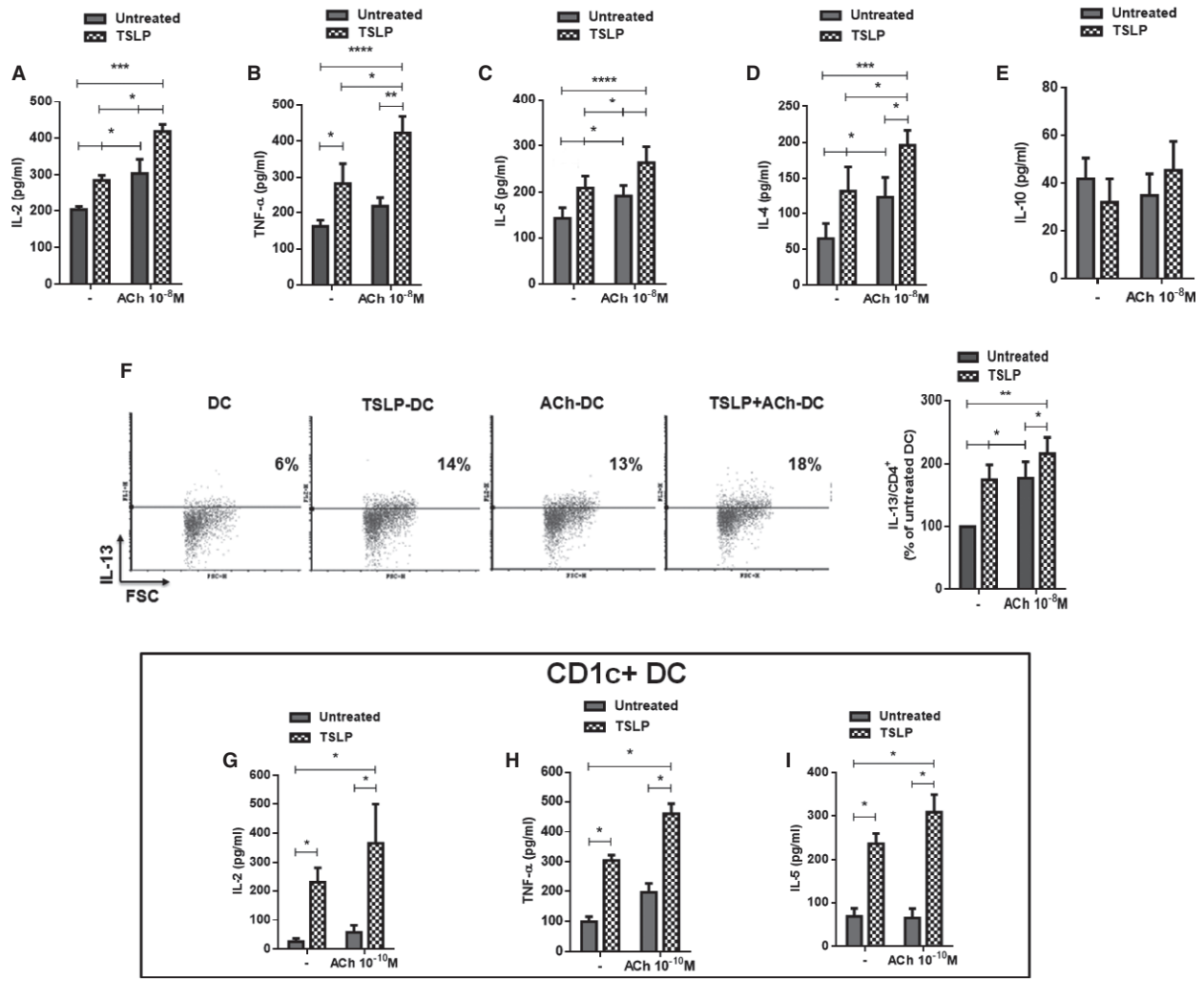


Figure 6 ACh increases the ability of TSLP-treated DC to induce the production of IL-2, TNF-α, IL-5, IL-4, and IL-13 by $CD4^+$ T cells in the course of MLR. DC (1×10^6/ml) were cultured for 18 h with or without ACh (10^{-8}M) and/or TSLP (15 ng/ml), washed and cultured with allogeneic naïve CD4+ T lymphocytes (DC/lymphocyte ratio 1 : 5) for 6 days. Then, cells were stimulated by PMA/ionomycin during 6 h and the production of cytokines was evaluated by ELISA (A-E) or by intracellular staining and flow cytometry (F). Results in panels (A-E and F right) represent the mean ± SEM of 5-8 experiments. Results in panel (F left) show a representative experiment ($n = 6$). (G-I) CD1c+ DC isolated from peripheral blood (1×10^6/ml) were cultured for 18 h with or without ACh (10^{-8}M) and/or TSLP (15 ng/ml), washed and cultured with allogeneic naïve CD4+ T lymphocytes (DC/lymphocyte ratio 1 : 5) for 6 days. Then, cells were stimulated by PMA/ionomycin during 6 h and the production of IL-2 (G), TNF-α (H), and IL-5 (I) was evaluated by ELISA. Results are expressed as the mean ± SEM of 3 experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

TNF-α and IL-8. This suggests that the modulatory action of ACh is exerted through muscarinic receptors.

To gain more information about the ability of ACh to promote the development of Th2 responses, we analyzed whether ACh-DC could stimulate the production of Th2 cytokines IL-4, IL-5, and IL-13 by $CD4^+$ T cells in the course of MLR. We found that ACh-DC stimulated the production of these three cytokines. Moreover, when DC were cultured with both TSLP and ACh, a higher stimulation of IL-4, IL-5, and IL-13 production was observed. These findings further suggest that ACh favors the acquisition of a Th2-promoting profile by DC.

Like other stimuli able to induce the activation and maturation of DC such as PAMPs and CD40L, TSLP has been shown to increase the expression of HLA-DR, CD80, CD86, and CD83 by DC (17,24,25). However, unlike PAMPs and CD40L, TSLP does not promote the production of Th1- or Th17-promoting cytokines, IL-12 and IL-23, respectively (17,18). Another important feature of TSLP-treated DC is their ability to produce chemokines able to recruit

neutrophils (IL-8) and Th2 cells (TARC and MDC) (17,24,26). Moreover, unlike classical Th2 cells which produce IL-4, IL-5, and IL-13, Th2 cells induced by TSLP-treated DC seem to acquire an inflammatory phenotype characterized by the production of these cytokines, together with TNF-α (16–18). These inflammatory Th2 cells appear to play an important role, not only in the development of allergic diseases such as asthma but also in the progression of cancer (27).

Our observations indicate that ACh stimulates a set of changes in DC identical to those induced by TSLP; an increased expression of OX40L, the upregulation of HLA-DR and CD83 expressions, the stimulation of the production of chemokines able to induce the recruitment of neutrophils and Th2 cells (IL-8, TARC, and MDC), and an increase in the production of IL-4, IL-5, IL-13, and TNF-α by $CD4^+$ T cells in the course of the MLR. Further experiments are needed to determine the relevance of ACh produced by both neuronal and non-neuronal cells, in the promotion of Th2 responses *in vivo*.

ACh represents the most important parasympathetic neurotransmitter in the airways (11). Because ACh induces airway smooth muscle contraction, anticholinergics are used as bronchodilators in obstructive airway diseases such as chronic pulmonary obstructive disease and asthma (11,13,28). However, it is increasingly accepted that anticholinergics not only prevent bronchoconstriction but also reduce mucus secretion, neutrophil infiltration, and airway remodeling (11,15,29). It is generally assumed that these protective effects involve the ability of anticholinergics to prevent the interaction of ACh with muscarinic receptors expressed by either smooth muscle cells or epithelial cells of the airway (30–32). Our present results suggest that anticholinergics could also protect the airway in the course of obstructive airway diseases by inhibiting the polarization of DC toward a Th2-promoting profile induced by ACh. Importantly, the immunomodulatory actions exerted by ACh could play a role, not only in asthma, but also in other allergic diseases. It is well known that the human skin contains a complete non-neuronal cholinergic system (33). Moreover, the levels of ACh markedly differ between healthy subjects and patients with atopic dermatitis, being the most marked differences found in the superficial layer of the skin. In fact, ACh was shown to be increased almost 14-fold in the eczematous skin of patients with atopic dermatitis compared with the healthy skin (34). We speculate that ACh could stimulate the ability of Langerhans cells not only to recruit Th2 cells, but also to stimulate the production of cytokines such as IL-4, IL-5, and IL-13, contributing to the development of allergic inflammation in the skin. Further observations, however, are needed to test this hypothesis.

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Conflict of interest

The authors declare that they have no conflict of interest.

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