

Activation of the cholinergic anti-inflammatory system by nicotine attenuates arthritis via suppression of macrophage migration

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Abstract. Activation of the cholinergic anti-inflammatory pathway (CAP), which relies on the alpha-7 nicotinic acetylcholine receptor, has been reported to reduce proinflammatory cytokine levels in experimental arthritis. To gain more insight regarding the role of the CAP in the pathogenesis of arthritis, the present study focused on the modulation of macrophage infiltration. In a mouse model of collagen-induced arthritis (CIA), nicotine and vagotomy were used to stimulate and inhibit the CAP, respectively. Subsequently, arthritic scores were measured and histopathological assessment of joint sections was conducted. Cluster of differentiation (CD)11b-positive macrophages in the synovium were studied by immunofluorescence histochemistry. The serum levels of chemokines, including macrophage inflammatory protein (MIP)-1 α , monocyte chemoattractant protein (MCP)-1 and MIP-2 were evaluated by ELISA. Furthermore, the expression levels of C-C chemokine receptor (CCR)2 and intercellular adhesion molecule (ICAM)-1 in the synovium were evaluated by immunohistochemical staining. The results indicated that treatment with nicotine significantly attenuated the clinical and histopathological changes associated with arthritis, reduced CD11b-positive macrophages in the synovium, and down-regulated the serum expression levels of MIP-1 α and MCP-1. Conversely, vagotomy aggravated arthritis and upregulated the expression levels of MCP-1. However, MIP-2 expression did not differ among the control, CIA, vagotomy and nicotine groups. In addition, the expression levels of CCR2 were reduced in the nicotine group; however, they were increased in the vagotomy group compared with in the untreated CIA group. The expression levels of ICAM-1 in the synovium were also influenced by activation of the CAP. Taken together, the

present results indicated that nicotine-induced activation of the CAP in mice with CIA may reduce the number of macrophages in the synovium, which may serve a role in alleviating arthritis in mice.

Introduction

Monocytes and macrophages are critical mediators of rheumatoid arthritis (RA) (1,2). High numbers of macrophages are present in inflamed tissues, particularly at the cartilage-pannus interface in human RA, and have been reported to be correlated with RA severity (3,4). In addition, inhibiting macrophage migration to sites of inflammation may markedly reduce inflammation and tissue destruction (5). Therefore, inhibiting the migration of macrophages is considered a potential key strategy for the treatment of RA. However, macrophage migration is a continuous and complex process, which includes rolling of monocytes along the endothelium, firm adhesion to the endothelium, and transendothelial cell migration. Within macrophage migration, chemotaxis and adhesion are considered the most important processes. Monocytes traverse the endothelium in order to enter developing and established lesions; this trafficking is directed by chemokines, such as macrophage inflammatory protein (MIP)-1 α , monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-8. C-C chemokine receptor (CCR)1, CCR2 and CXC chemokine receptor 2 are considered classical chemokine receptors, which have been proposed to affect monocyte recruitment in mouse models of arthritis (6-8). In addition, the firm adhesion of monocytes is mediated via the endothelial expression of members of the immunoglobulin superfamily, such as intercellular adhesion molecule (ICAM)-1, and their counterligands, which are expressed by leukocytes, including cluster of differentiation (CD)11b/CD18.

The cholinergic anti-inflammatory pathway (CAP), which transmits information from the brain to the peripheral immune system via the parasympathetic nervous system, was initially described in 2000 (9). Cholinergic stimulation, by vagus nerve electrical stimulation or treatment with selective cholinergic agonists, suppresses the production of cytokines in preclinical models of systemic inflammation, including endotoxemia, hemorrhagic shock, ischemia-reperfusion injury and acute lung injury (9-11). Macrophages express alpha-7 nicotinic acetylcholine receptor, pharmacological stimulation

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of which reduces the production of inflammatory cytokines by macrophages (12,13). In previous experiments by authors of the present study, and others, the CAP has been revealed to exert a protective effect on RA (14-16); the underlying mechanism may be associated with the inhibition of T helper (Th)17 cell responses and may improve the Th1/Th2 imbalance in collagen-induced arthritis (CIA). However, the specific mechanisms remain unclear.

The present study established that reduced numbers of macrophages are present in synovial tissues following activation of cholinergic signaling, since synovial tissues are not innervated by the vagus nerve. The present study aimed to elaborate the protective effects of the CAP on RA and to elucidate its cellular molecular mechanisms, thus providing an experimental basis regarding how the CAP regulates inflammatory and immune responses in RA.

Materials and methods

Mice. A total of 56 male DBA/1 mice (weight, 20 g; age, 6-8 weeks; 24 mice died prematurely whilst the model was established) were purchased from the SJA Laboratory Animal Co. (Shanghai, China) for use in the CIA studies. The mice were housed under specific-pathogen-free conditions at 24-28°C, 40-70% humidity, 12 h light/dark cycle and free access to food and water. The animal experiments were performed in accordance with the institutional guidelines for animal care, and were approved by the committee for the use and care of animals at Central South University (Changsha, China).

Experimental groups. The mice were randomly divided into four groups (n=8): Control group [sham vagotomy + phosphate-buffered saline (PBS)], model group (sham vagotomy + CIA + PBS), vagotomy group (vagotomy + CIA + PBS), and nicotine group (sham vagotomy + CIA + nicotine). To inhibit the CAP, mice in the vagotomy group were subjected to left-side cervical vagotomy 4 days prior to CIA induction (14). In sham-operated mice, the left vagus nerve was exposed and isolated from the surrounding tissue but was not transected. In the nicotine group, the peripheral segment of the CAP was stimulated by intraperitoneal pretreatment with nicotine daily (250 µg/kg; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), beginning 4 days prior to CIA induction. The other groups were injected with PBS as a control.

Induction and evaluation of CIA. CIA was induced in the DBA/1 mice as previously described (14). Bovine type II collagen (2 mg/ml; Chondrex, Inc., Redmond, WA, USA) was emulsified in an equal volume of Freund's complete adjuvant (containing 4 mg/ml *Mycobacterium tuberculosis*; Chondrex, Inc.) at 4°C by magnetic stirring until fully emulsified. The final concentration of collagen was 1 mg/ml. All male DBA/1 mice, with the exception of the control mice, were initially immunized intracutaneously at the base of the tail with 0.1 ml of this emulsion (100 µg collagen). On day 21, the mice were administered booster injections of 0.1 ml emulsion in Freund's incomplete adjuvant (Chondrex, Inc.) in the same manner.

Clinical signs of arthritis in the joints were visually assessed every 3 days by two observers. All animals were regularly examined for signs of arthritis, and the disease severity for each paw was graded on a scale between 0 and 4, according to the levels of erythema, swelling and induration. Arthritis index score criteria were as follows: 0, no redness or swelling of the joints; 1, red or slight swelling; 2, moderate swelling; 3, severe swelling; and 4, severe swelling and unloading. Assessment of incidence and macroscopic score were carried out by two independent observers, without knowledge of the experimental groups. The total arthritic score per mouse was derived from the sum of the individual scores of four paws. Two independent observers without knowledge of the experimental groups assessed the incidence and macroscopic score.

Immunohistochemical evaluation of CIA. The mice were sacrificed by cervical dislocation on day 42, and their hind paws were collected. The joint tissues were fixed in 4% paraformaldehyde overnight at room temperature, decalcified in 13% ethylenediamine tetra-acetic acid for 2-3 weeks and embedded in paraffin and cut into 5 µm sections. The sections (2.5x2.5 cm) were then dewaxed using xylene, dehydrated using an alcohol gradient, and were stained with hematoxylin and eosin (H&E) for 1 min at room temperature for histological assessment. Arthritis severity in histological samples was determined by cumulative assessment of synovial inflammation. The scoring was: 0, normal; 1, minimal; 2, mild; 3, moderate; 4, marked; and 5, severe. This was completed by two independent observed, without knowledge of the experimental groups. Histopathological sections of the hind paws were examined for hyperplasia of the synovial membrane, infiltration with mononuclear cells, and cartilage and bone damage (14).

For immunofluorescence detection of macrophage infiltration, sections were dewaxed and dehydrated as aforementioned. Slides were retrieved using a heat-mediated retrieval method, and endogenous peroxidase activity was quenched with 3% H₂O₂ for 5 min at room temperature. The sections were then blocked with 3% bovine serum albumin (Sijiqing, Hanzhou, China) for 1 h at room temperature and were incubated overnight at 4°C with an anti-CD11b primary antibody (1:200; cat. no. 101213; BioLegend, San Diego, CA, USA). Subsequently, the sections were incubated with Alexa Fluor 555-conjugated goat anti-rat immunoglobulin G (IgG; cat. no. A-21434; 1:150; Molecular Probes; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 1 h at room temperature. The number of Alexa Fluor 555-labeled CD11b-positive macrophages was counted under a confocal microscope (LSM780; Carl Zeiss Jena GmbH, Jena, Germany) using a x63 oil immersion objective by a blinded observer.

For staining of CCR2 and ICAM-1, the sections were pretreated with proteinase K for CCR2 retrieval for 20 min at 37°C, and were then treated with citric buffer (pH 6) for heat-mediated antigen retrieval of ICAM-1 at 80°C for 20 min. Sections were incubated with rabbit anti-mouse CCR2 (1:200; cat. no. ab32144; Abcam, Cambridge, MA, USA), rabbit anti-mouse ICAM-1 monoclonal antibody (1:400; cat. no. ab124759; Abcam) or normal rabbit IgG (1:200; cat. no. A7016; Biyuntian, Shanghai, China) at 4°C overnight. Subsequently, the samples were washed three

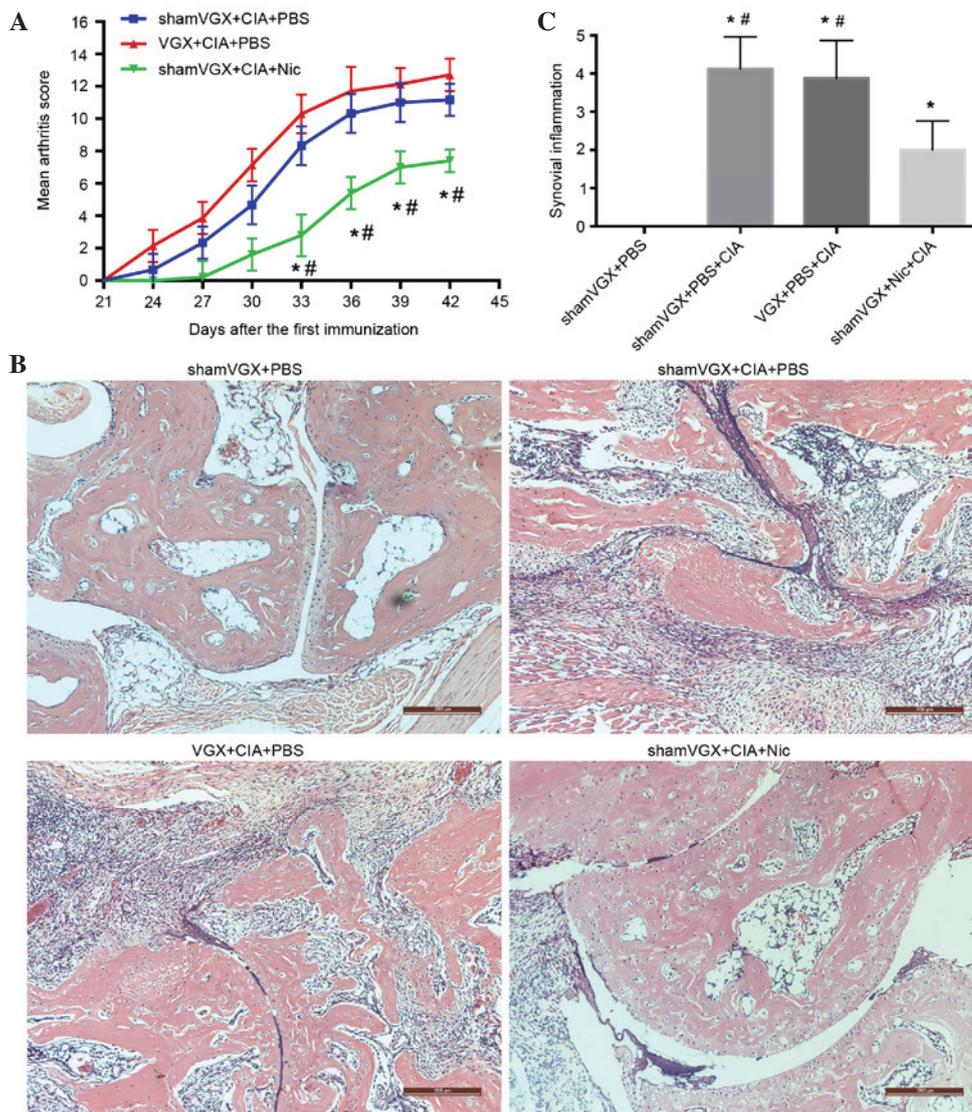


Figure 1. Reduced severity and incidence of CIA in mice treated with Nic. The mice were immunized with bovine type II collagen to establish CIA. A total of 4 days prior to the first immunization, mice were either intraperitoneally infused with Nic (250 $\mu\text{g}/\text{kg}$), which continued every day until sacrifice, or underwent left-side cervical VGX. PBS served as the control. (A) Arthritic scores were recorded. Data are presented as the mean \pm standard deviation (n=8 mice/group). * $P<0.05$ vs. the shamVGX + CIA + PBS group; # $P<0.05$ vs. the VGX + CIA + PBS group. (B and C) Mice were sacrificed and subjected to a histopathological examination. Infiltration of inflammatory cells into the synovial tissue was (B) detected and (C) scored (magnification, x100). Synovial inflammation was scored 0-5. Data are presented as the mean \pm standard deviation (n=8 mice/group). * $P<0.05$ vs. the shamVGX + PBS group; # $P<0.05$ vs. the shamVGX + Nic + CIA group. CIA, collagen-induced arthritis; VGX, vagotomy; Nic, nicotine; PBS, phosphate-buffered saline.

times for 5 min in PBS and were incubated with biotin-conjugated goat anti-rabbit IgG (ABC kit; cat. no. PK-6100; Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature. After three 5-min washes in PBS, the sections were incubated with an avidin-biotinylated enzyme complex (ABC kit; Vector Laboratories) for 2 h at room temperature. Positive signals were visualized using a diaminobenzidine kit (Vector Laboratories), the sections were counterstained with hematoxylin, images were captured using an Olympus microscope (IMT-2, Olympus Corporation, Tokyo, Japan) and were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Chemokine quantification by ELISA. On experimental day 42, the mice were sacrificed, and serum samples were harvested. Whole blood samples (0.8-1 ml each) were centrifuged at

12,000 $\times g$ for 20 min at 4°C. The serum levels of MIP-1 α (cat. no. MMA00; R&D Systems, Inc., Minneapolis, MN, USA), MCP-1 (cat. no. 81-BMS6005; eBioscience, Inc., San Diego, CA, USA) and MIP-2 (cat. no. 70-EK21422; Multiscience Biotech, Co., Ltd., Hangzhou, China) were measured using ELISA kits, according to the manufacturers' protocols.

Isolation of mononuclear cells and flow cytometry. Spleen samples were obtained from the sacrificed mice. Splenocytes were separated from the spleen using lymphocyte-separation medium (Histopaque[®]-1119; Sigma-Aldrich; Merck Millipore) after filtering the samples with a cell strainer (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, the cells were incubated for 1 h at 4°C with phycoerythrin-conjugated anti-CD11b (eBioscience, Inc.) or the respective isotype control (cat. no. 12-4031; eBioscience, Inc.). The cells were

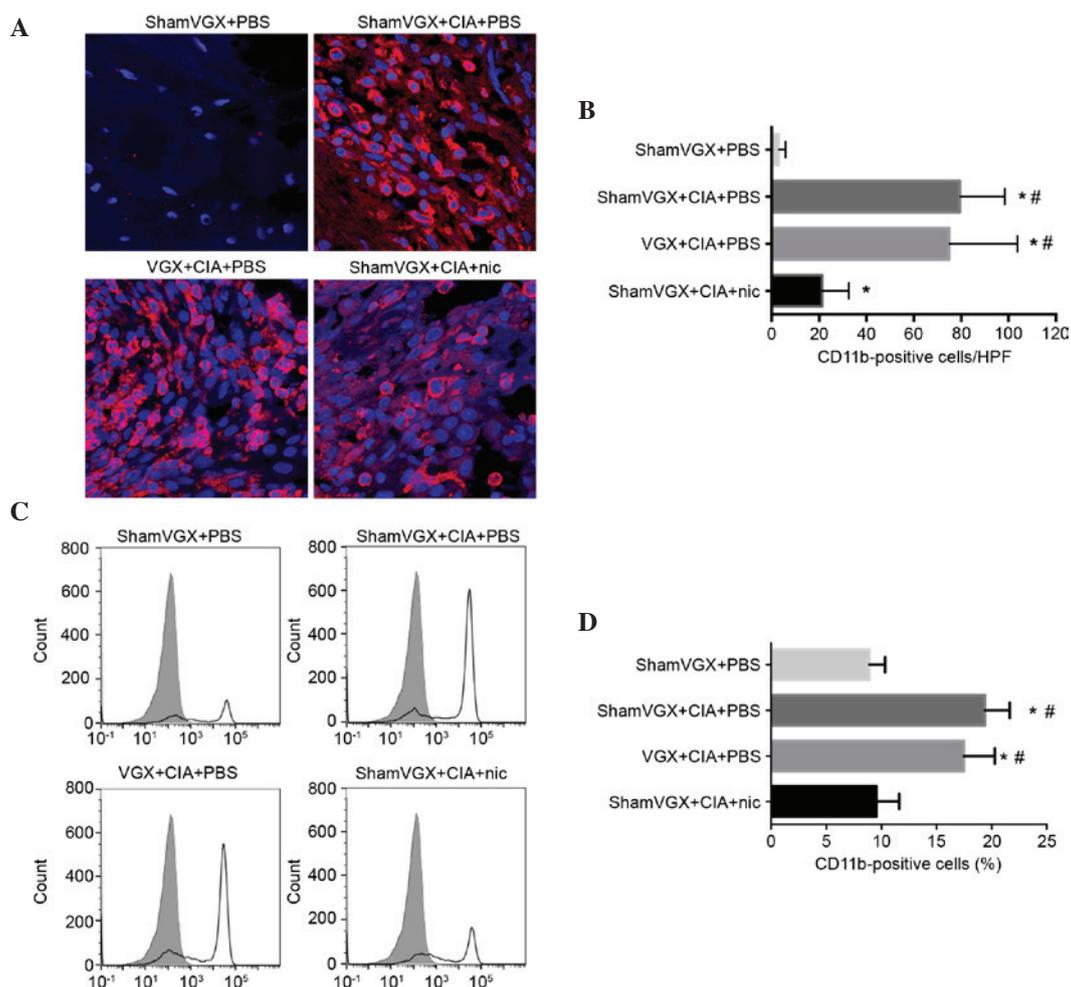


Figure 2. Nic reduced the migration of CD11b-positive macrophages into the synovium and spleen. After 42 days, the mice were sacrificed, and their ankle joints and spleen tissues were harvested and examined for migrated macrophages. (A) Immunofluorescence staining was used to detect macrophages in the ankle joints of the mice. CD11b-positive macrophages were labeled with Alexa Fluor 555. (B) Average number of CD11b-positive cells in the synovial sections from the four groups. Data are presented as the mean \pm standard deviation (n=8 mice/group). (C) Single cells were separated from the spleen cells of the mice, labeled with PE, and detected by flow cytometry. Solid lines indicate staining with PE-labeled anti-CD11b antibodies; shaded histograms indicate staining with respective isotype antibodies. (D) Percentage of CD11b-positive cells. Data are presented as the mean \pm standard deviation (n=8 mice/group). *P<0.05 vs. the shamVGX + PBS group; #P<0.05 vs. the shamVGX + CIA + Nic group. CIA, collagen-induced arthritis; VGX, vagotomy; Nic, nicotine; PBS, phosphate-buffered saline; CD11b, cluster of differentiation 11b; PE, phycoerythrin.

then washed with PBS and resuspended in 100 μ l PBS for flow cytometric analysis (BD FACS Canto II; BD Biosciences). The data were analyzed using FlowJo software version 9.9.4 (FlowJo, LLC, Ashland, OR, USA).

Statistical analysis. Data were analyzed using GraphPad Prism software version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) and are presented as the mean \pm standard deviation. The experiments were repeated twice. The significance of differences between groups was determined by one-way analysis of variance followed by a multiple comparisons test (Student-Newman-Keuls). The Kruskal-Wallis test was used to determine the heterogeneity of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Nicotine attenuates the clinical and histopathological changes associated with CIA. The CIA model was induced as

mentioned. The arthritis index score was recorded by two researchers every 3 days after the second immunization until the mice were sacrificed. Slight swelling could be observed in the vagotomy group beginning at day 24, and reaching a peak at day 42. The model group began to present with arthritic symptoms at day 27, with symptoms peaking after 42 days. Arthritis developed slowly and was milder in the nicotine group (Fig. 1A). The histopathological features of the four groups were assessed by H&E staining. The results were consistent with the aforementioned findings, in that the histopathological changes in the nicotine group were reduced, as detected by decreased synovial proliferation, inflammatory cell infiltration and bone destruction (Fig. 1B). Semi-quantitative analysis of the histopathological features is presented in Fig. 1C.

Nicotine reduces macrophage infiltration in the synovium and spleen of CIA mice. Since the present study suggested that nicotine attenuates clinical arthritis and restrains cytokine production, such as TNF- α in our previous study (14), it was

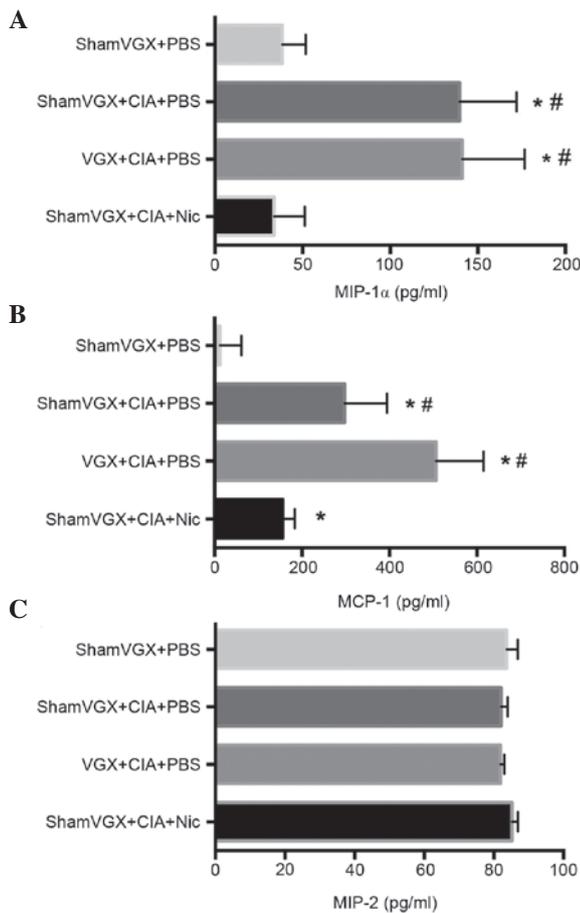


Figure 3. Effects of Nic on chemokine levels in the serum of mice. Mice were immunized with bovine type II collagen to establish CIA. Vagotomy or nicotine treatment was conducted 4 days prior to the first immunization. On day 42 post-immunization, serum was collected for ELISA. (A) MIP-1 α , (B) MCP-1 and (C) MIP-2 levels were measured by ELISA. Results are presented as the mean \pm standard deviation (n=8 mice/group). *P<0.05 vs. the shamVGX + PBS group; #P<0.05 vs. the shamVGX + CIA Nic group. There was no differences among the four groups in terms of MIP-2 expression. CIA, collagen-induced arthritis; VGX, vagotomy; Nic, nicotine; PBS, phosphate-buffered saline; MIP, macrophage inflammatory protein; MCP-1, monocyte chemoattractant protein-1.

determined whether nicotine can modulate macrophage infiltration in the synovium and spleen. Macrophage infiltration serves a critical role in modulating the immune responses. In mice, the CD11b antigen, as a marker of monocytes/macrophages, is highly expressed on monocytes, macrophages, and to a lesser extent, on granulocytes and a subset of dendritic cells. The present study detected the distribution of CD11b-positive macrophages in the synovium and spleen. Immunofluorescence analysis detected an increase in infiltrated CD11b-positive macrophages in the synovium of the model, vagotomy and nicotine groups, as compared with in the control group. However, among the model, vagotomy and nicotine groups, the percentage of CD11b-positive macrophages was lowest in the nicotine group (Fig. 2A and B). In addition, the percentage of CD11b-positive cells was detected among the splenic mononuclear cells in the four groups, using flow cytometry (Fig. 2C and D). In the model group, the percentage of CD11b-positive cells was increased compared with in the control group. Treatment with nicotine significantly reduced the percentage

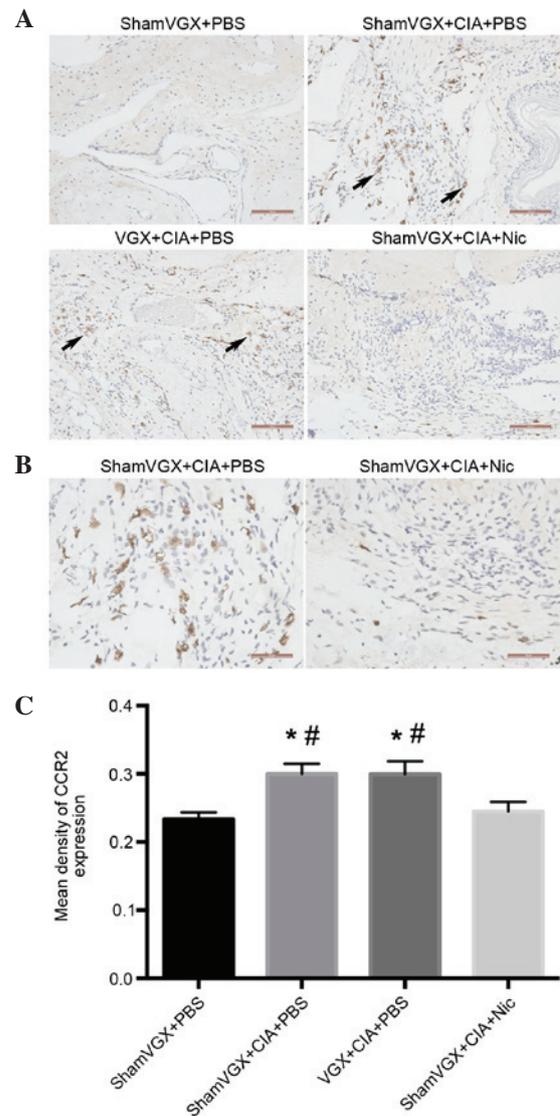


Figure 4. Administration of nicotine reduced the expression of CCR2 in the synovium of CIA mice. (A and B) Immunohistochemical evaluation of synovial tissue, in which the brown color represents CCR2-positive cells (A, x200 magnification; black arrow, CCR2-positive cells; B, x400 magnification). (C) Mean density of CCR2 expression in synovial sections from the four groups analyzed by Image-Pro Plus 6.0 software. Data are presented as the mean \pm standard deviation (n=6 mice/group). *P<0.05 vs. the shamVGX + PBS group; #P<0.05 vs. the shamVGX + CIA Nic group. CIA, collagen-induced arthritis; VGX, vagotomy; Nic, nicotine; PBS, phosphate-buffered saline; CCR2, C-C chemokine receptor type 2.

of CD11b-positive cells in the spleen, as compared with in the model and vagotomy groups.

Nicotine suppresses the serum levels of MIP-1 α and MCP-1, but not MIP-2. Chemokines serve a key role in the process of leukocyte extravasation. To determine whether the CAP affects chemotaxis of macrophages in the CIA model, the present study detected the expression levels of the macrophage-associated chemokines MIP-1 α , MCP-1 and MIP-2, which have been reported to have major roles in the trafficking of monocytes toward synovial tissue, in the serum of the four groups by ELISA (7,17). Compared with its release in the model and vagotomy groups, nicotine, a classical cholinergic agonist, significantly attenuated release of the

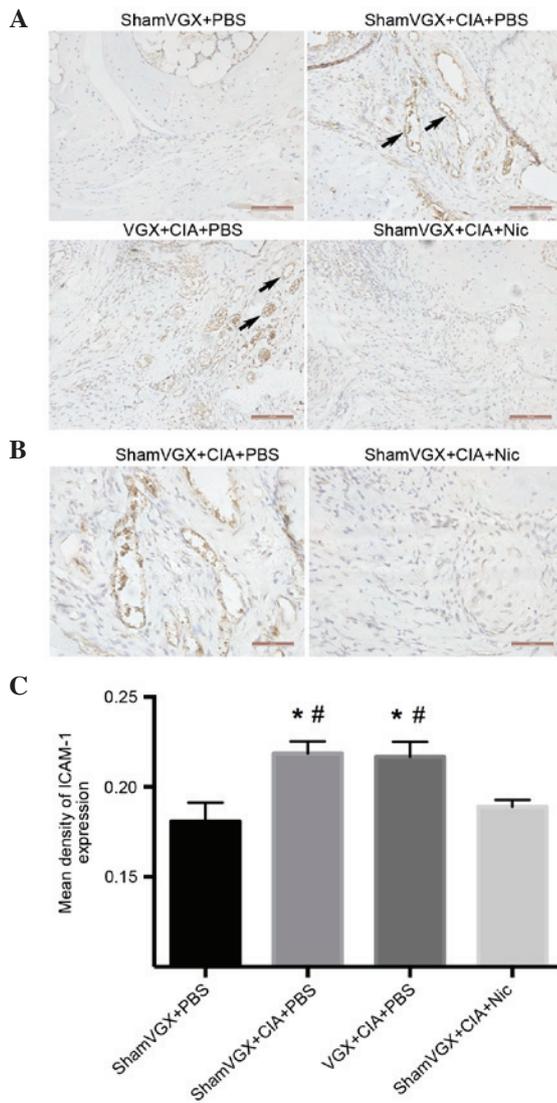


Figure 5. Reduced ICAM-1 expression in synovial tissues. (A and B) Immunohistochemistry was used to analyze ICAM-1 expression in the synovial tissues of mice. Positive cells were detected by the avidin-biotinylated enzyme complex technique with diaminobenzidine development (A, x200 magnification; B, x400 magnification; black arrow, ICAM-1-positive cells). (C) Mean density of ICAM-1 expression in synovial sections from the four groups analyzed by Image-Pro Plus 6.0 software. Data are presented as the mean \pm standard deviation ($n=6$ mice/group). * $P<0.05$ vs. the shamVGX + PBS group; # $P<0.05$ vs. the shamVGX + CIA Nic group. CIA, collagen-induced arthritis; VGX, vagotomy; Nic, nicotine; PBS, phosphate-buffered saline; ICAM-1, intercellular adhesion molecule-1.

chemokines MIP-1 α and MCP-1 (Fig. 3A and B), but not MIP-2 (Fig. 3C).

Nicotine treatment results in reduced CCR2 expression in the synovium of CIA mice. Chemokines control the directed movement of cells expressing their respective receptors. The chemokine receptor CCR2 is bound by MCP-1, thus contributing to the migration of monocytes from the bloodstream into the tissue (18). The present study examined CCR2 expression in the synovial tissue by immunohistochemistry. The results of a semi-quantitative analysis indicated that CCR2 was highly expressed on synoviocytes in mice with CIA but not in normal mice, and nicotine treatment reduced CCR2 expression in the CIA mice (Fig. 4A-C).

Nicotine decreases endothelial cell surface levels of ICAM-1 in CIA mice. ICAM-1 has classically been considered to serve a role in intercellular adhesion. To evaluate the effects of nicotine on the expression of adhesion molecules on vascular endothelial cells, ICAM-1 production was measured in the synovium. Immunohistochemical analysis of mouse synovial sections revealed that ICAM-1 was highly expressed in the model and vagotomy groups. Conversely, ICAM-1 expression was significantly suppressed in nicotine-treated mice (Fig. 5A-C).

Discussion

Vagally mediated anti-inflammatory action, also known as the CAP, was clearly described by Tracey (19). In previous years, several researches have reported that activation of the CAP suppresses the clinical and histological manifestations of CIA in mice with established disease (14-16,20,21). The underlying mechanisms may be associated with downregulation of the expression of tumor necrosis factor- α and IL-6, inhibition of Th17 cell responses and improvement in the Th1/Th2 imbalance in CIA; however, the specific mechanism underlying the regulatory effects of nicotine on RA remains complex and only partially understood.

Notably, the present results revealed that nicotine treatment reduced the number of CD11b-positive macrophages in the synovium and spleen samples of CIA mice. Synovial macrophages participate in several of the events that direct inflammation, including angiogenic stimulation, leukocyte and lymphocyte recruitment, fibroblast proliferation, and protease secretion, thus resulting in joint destruction (3,22,23). Synovial macrophages are currently considered the most reliable biomarker for disease severity and therapeutic response in RA (24). Therefore, the present findings suggested that the reduced infiltration of macrophages mediated by nicotine may serve an important role in protection against autoimmune arthritis. However, in the present study, the mice in the vagotomy group were subjected to left-side cervical vagotomy 4 days prior to CIA induction, the vagotomy did not exacerbate CIA-associated inflammation, which may be attributed to a compensatory role for the other side of the vagus nerve. This finding was consistent with the results of previous studies (15,16).

Macrophage infiltration is a process that involves complex and consecutive changes. Monocyte transmigration through endothelial monolayers is directed by chemotaxis, which is a crucial step in the process of complete monocyte recruitment to the vascular wall. The present study demonstrated that nicotine treatment in a CIA model reduced the production of MIP-1 α and MCP-1. MIP-1 α , which belongs to the CC chemokine family, is chemotactic for monocytes and lymphocytes in RA. MIP-1 α may be considered one of the major cytokines that contributes to the chemoattraction and retention of RA macrophages in inflamed joints (25). MCP-1, another CC chemokine, was initially identified as a monocyte-specific chemoattractant, and has also been reported to serve a role in T-cell differentiation and angiogenesis (26), which may be important in RA pathogenesis. Treatment with an MCP-1 antagonist has been reported to ameliorate disease severity in adjuvant-induced arthritis by decreasing macrophage

infiltration (27). However, in the present study, there were no significant differences in MIP-2 levels among the sera. MIP-2, which is a CXC chemokine (and is known as the murine equivalent of CXC chemokine ligand 8/IL-8), was among the first chemokines reported to be involved in leukocyte chemotaxis. MIP-2 has also been demonstrated to serve a major role in neutrophil and monocyte trafficking toward the synovial tissue (7,28). The results of the present study suggested that the action of nicotine may not depend on the production of MIP-2 in the CIA model.

CCR2 is the major chemokine receptor used to classify monocyte subsets (29), and is the predominant functional receptor for MCP-1. MCP-1-CCR2 signaling may activate p42/44 mitogen-activated protein kinases (MAPK) and protein kinase C (PKC) through G proteins, in order to regulate cellular adhesion and motility in macrophages (30). Based on previous data, ~50% of infiltrating cells in the synovial tissue are CCR2-positive monocytes (31). While investigating whether activation of the CAP also influences CCR2 expression in the synovium, the present study demonstrated that nicotine treatment reduced CCR2 expression, as determined by immunohistochemical semi-quantitative analysis. These results indicated that the interaction between MCP-1/C-C chemokine ligand (CCL)2 and CCR2 may promote macrophage migration into the synovium during CIA. A previous study demonstrated that CCL2-CCR2 signaling may activate p42/44 MAPK and PKC through G proteins to regulate cellular adhesion and motility in macrophages (30). Our studies, however, do not rule out the possibility of more direct evidence of macrophage migration in synovial tissues. Further investigations are required to clarify this point. Other interactions between chemokines and their receptors, such as between MIP-1 α /CCL3 and CCR1, may also contribute to macrophage migration (32). In addition, a previous study revealed that a CCR1 antagonist, which reduces macrophage accumulation in RA synovium, is effective for treating RA (33).

ICAM-1 is an adhesion molecule expressed by activated endothelial cells, which is believed to mediate leukocyte migration across the endothelium. However, the potential effects of nicotine on adhesion molecule expression remain controversial. Takahashi *et al* reported that nicotine inhibits IL-18-enhanced expression of ICAM-1 on monocytes (34). Conversely, Cirillo *et al* and others demonstrated that nicotine promotes ICAM expression on endothelial cells (35,36). The present study revealed that stimulation of the CAP reduced production of ICAM-1, not only on endothelial cells but also on leukocytes in the peripheral blood (Fig. 5A). Therefore, based on this finding, it may be hypothesized that the CAP has a dominant influence on monocyte adhesion to endothelial cells.

In conclusion, the results of the present study indicated that activation of the CAP via nicotine stimulation reduces CIA-associated inflammation by decreasing the number of macrophages in synovial tissues, which is mediated by effects not only on the chemotaxis of macrophages but also on macrophage adhesion to endothelial cells. These observations suggest that stimulation of cholinergic signaling potentially serves a key role in initiating and maintaining joint inflammation in patients with RA. Therefore, further investigation regarding the role of CAP in this context is required.

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