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C_3Mab-2: An Anti-Mouse CCR3 Monoclonal Antibody for Immunocytochemistry

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The C–C motif chemokine receptor 3 (CCR3) is a G protein-coupled receptor activated by eotaxin-1-3, MCP-2-4, and RANTES. CCR3 is associated with allergic diseases and cancer development and is highly expressed in eosinophils, basophils, and cancer cells. Besides, research on the physiological roles of CCR3 is ongoing. Thus, specific monoclonal antibodies (mAbs) for CCR3 would be useful for diagnostic and therapeutic purposes and for unraveling the function of CCR3. We previously developed an anti-mouse CCR3 (mCCR3) mAb (C_3Mab-2; rat IgG_{2b}, kappa) using the Cell-Based Immunization and Screening method and showed that C_3Mab-2 could detect endogenous and exogenous mCCR3 in flow cytometry. In this study, we showed that C_3Mab-2 and its recombinant antibody (recC_3Mab-2f) specifically recognized endogenous mCCR3 in P388 (a mouse lymphocyte-like cell line) and J774-1 (a mouse macrophage-like cell line) cells and are usable in immunocytochemistry.

Keywords: mouse CCR3, C_3Mab-2, monoclonal antibody, immunocytochemical analysis

Introduction

THE C–C MOTIF CHEMOKINE receptor 3 (CCR3) is a seven-transmembrane G protein-coupled receptor (GPCR) activated by eotaxin-1/CCL11, eotaxin-2/CCL24, eotaxin-3/CCL26, MCP-2/CCL8, MCP-3/CCL7, MCP-4/CCL13, and RANTES/CCL5.[1] CCR3 is expressed by eosinophils, basophils, T helper 2 (T_H2) cells, and airway smooth muscle cells[2–5]; it contributes to the recruitment/migration of cells at allergic inflammation and asthma sites. Moreover, CCR3 participates in cancer development. Cancer cells from breast cancer, glioblastoma, lung adenocarcinoma, and renal cell carcinoma express high CCR3 levels, increasing their proliferation, migration, and invasion.[6–9] Therefore, CCR3 can be a diagnostic and therapeutic target molecule for allergic diseases and cancer.

Developing diagnostic and therapeutic methods requires a precise understanding of the physiological roles of CCR3. However, the physiological roles of CCR3 remain unclear. In general, monoclonal antibodies (mAbs) help explore diagnostic and therapeutic methods and elucidate the functions of target molecules. Therefore, developing mAbs that specifically recognize endogenous CCR3 in multiple applications would be advantageous, although the production of mAbs for GPCR remains notoriously difficult.

Using the Cell-Based Immunization and Screening (CBIS) method, we have developed mAbs that target transmembrane proteins, such as podoplanin,[10,11] CD133,[12] EpCAM,[13] PD-L1,[14] and several GPCRs, such as CCR8[15] and CCR9.[16] We also previously developed an anti-mouse CCR3 (mCCR3) mAb (C_3Mab-2; rat IgG_{2b}, kappa) through the CBIS method and showed that C_3Mab-2 was usable in flow cytometry.[17] Furthermore, this study demonstrates that C_3Mab-2 is usable in immunocytochemistry against endogenous mCCR3 in cultured lymphocyte-like cells and macrophage-like cells.

Materials and Methods

Cell lines

We purchased Chinese hamster ovary (CHO)-K1 cells from the American Type Culture Collection (Manassas, VA).

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We previously reported the development of the CHO-K1 cell line stably expressing mCCR3 (CHO/mCCR3).[17] The Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan) provided the mouse lymphocyte-like cell line (P388 cells) and mouse macrophage-like cell line (J774-1 cells). We cultured the CHO-K1, CHO/mCCR3, P388, and J774-1 cells in Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Nacalai Tesque, Inc.). We maintained the cells in a humidified atmosphere at 37°C with 5% CO_2.

Antibodies

We previously described C_3Mab-2 development.[17] To produce recombinant C_3Mab-2 (recC_3Mab-2f), we subcloned variable (V_H) and constant (C_H) regions of heavy chain cDNAs of C_3Mab-2 into the pCAG-Neo vector along with variable (V_L) and constant (C_L) regions of light chain cDNAs of C_3Mab-2 into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). We transfected C_3Mab-2 vectors into BINDS-09 cells (www.med-tohoku-antibody.com/topics/001_paper_cell.htm) using the ExpiCHO Expression System (Thermo Fisher Scientific, Inc.). We purified the resulting mAb (recC_3Mab-2f) using an Ab-Capcher (ProteNova, Kagawa, Japan). We purchased an anti-mCCR3 mAb (clone J073E5) from BioLegend (San Diego, CA) and Alexa Fluor 488-conjugated anti-rat IgG from Cell Signaling Technology, Inc., (Danvers, MA).

Immunocytochemistry

We conducted immunocytochemistry in two ways: a conventional way (prefix method) and a modified way (postfix method). In the prefix method, we fixed CHO-K1, CHO/mCCR3, P388, and J774-1 cells with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 minutes

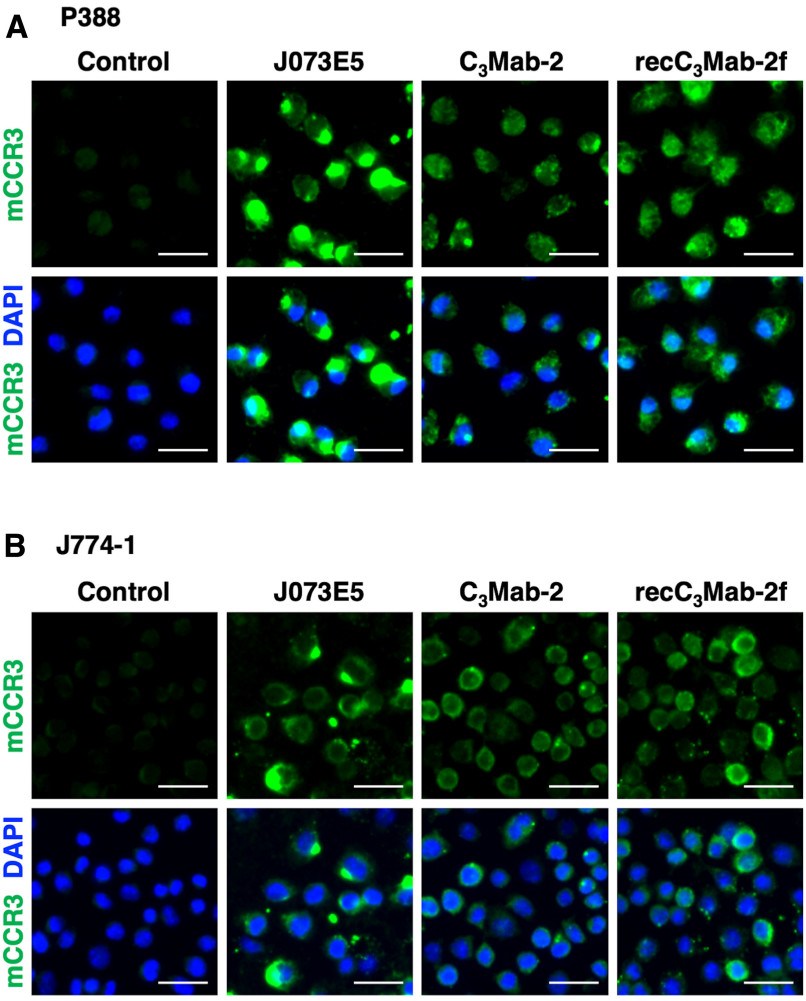

FIG. 1. Immunocytochemical experiment against endogenous mCCR3 through the prefix method. P388 cells **(A)** and J774-1 cells **(B)** fixed with 4% PFA were incubated with buffer control, 10 µg/mL of J073E5, 10 µg/mL of C_3Mab-2, or 10 µg/mL of recC_3Mab-2f for 1 hour, followed by incubation with Alexa Fluor 488-conjugated anti-rat IgG and DAPI for 45 minutes. Scale bars: 20 µm. DAPI, 4′,6-diamidino-2-phenylindole; mCCR3, mouse C–C motif chemokine receptor 3; PFA, paraformaldehyde.

and quenched with 50 mM NH_4Cl in PBS supplemented with 0.2 mM Ca^{2+} and 2 mM Mg^{2+} (PBSc/m) for 10 minutes at room temperature. Next, we blocked the cells using blocking buffer (PBSc/m supplemented with 0.5% bovine serum albumin) for 30 minutes and incubated them with 10 μg/mL of primary antibodies for 1 hour and then with Alexa Fluor 488-conjugated anti-rat IgG (1:400) for 45 minutes at room temperature. We stained the cell nuclei with 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Inc.).

In the postfix method, we blocked the CHO-K1, CHO/mCCR3, P388, and J774-1 cells using a chilled blocking buffer for 30 minutes on ice. We further incubated the cells with chilled primary antibodies (10 μg/mL) for 1 hour, followed by chilled secondary antibody (1:400) for 45 minutes on ice. We then fixed the cells with 4% PFA in PBS for 10 minutes on ice and an additional 15 minutes at room temperature. Finally, we treated the cells with PBSc/m supplemented with DAPI for 10 minutes at room temperature. We acquired the fluorescence images using a 40× objective on a BZ-X800 digital microscope (Keyence, Osaka, Japan).

Results

Immunocytochemical analysis of CHO/mCCR3 cells using C_3Mab-2 and recC_3Mab-2f

We previously showed that C_3Mab-2 allowed to weakly detect CHO/mCCR3 cells using flow cytometry.[17] First, to investigate the binding of C_3Mab-2 (10 μg/mL) and recC_3Mab-2f (10 μg/mL) to exogenous mCCR3, we fixed CHO/mCCR3 cells in 4% PFA and conducted immunocytochemistry (prefix method). Unexpectedly, C_3Mab-2 and recC_3Mab-2f did not detect exogenous mCCR3 in CHO/mCCR3 cells (Supplementary Fig. S1A). Both antibodies showed no signal in the parental CHO-K1 cells (Supplementary Fig. S1B). By contrast, a commercially available anti-mCCR3 antibody (J073E5, 10 μg/mL) clearly detected the mCCR3 (Supplementary Fig. S1A), but it showed strong nonspecific signals in CHO-K1 cells (Supplementary Fig. S1B).

We wondered whether the PFA fixation changed the structure of cell surface proteins in CHO-K1 cells, causing the nonspecific signals observed with J073E5. To address this

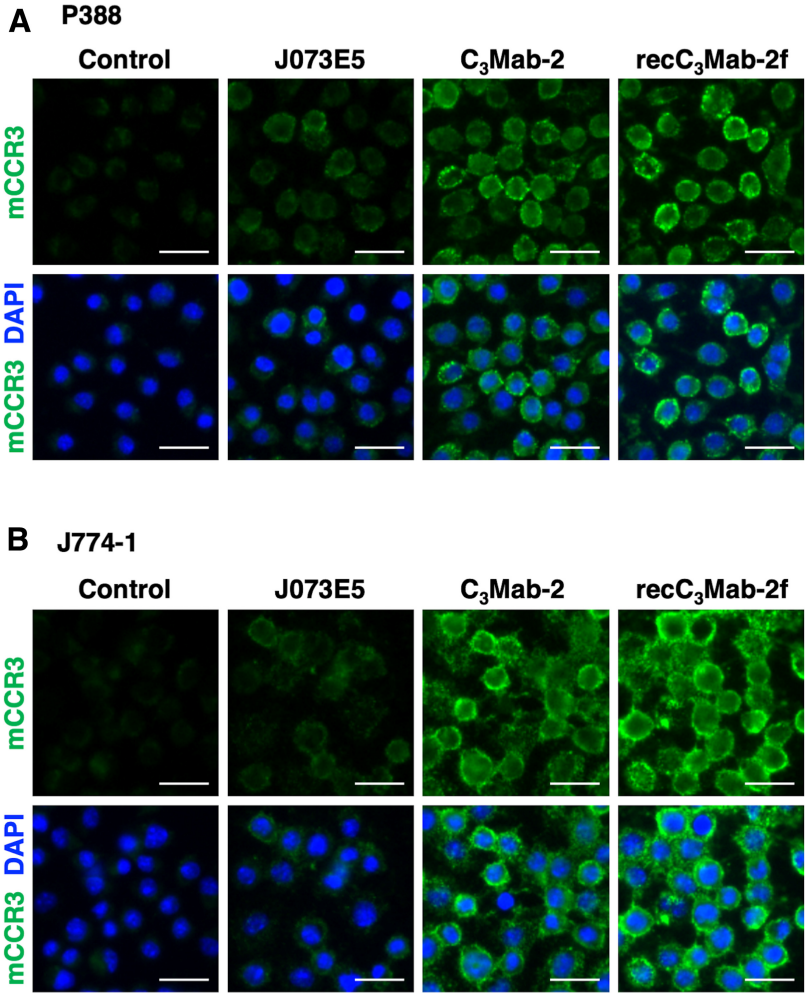

FIG. 2. Immunocytochemical experiment against endogenous mCCR3 through the postfix method. P388 cells **(A)** and J774-1 cells **(B)** were incubated with buffer control, 10 μg/mL of J073E5, 10 μg/mL of C_3Mab-2, or 10 μg/mL of recC_3Mab-2f for 1 hour, followed by incubation with Alexa Fluor 488-conjugated anti-rat IgG for 45 minutes on ice. The cells were fixed with 4% PFA and stained with DAPI. Scale bars: 20 μm.

issue, we labeled living cells with J073E5 on ice and the corresponding secondary antibody before fixation with 4% PFA (postfix method). Consequently, J073E5 recognized mCCR3 in CHO/mCCR3 cells (Supplementary Fig. S2A) without visible nonspecific signals in CHO-K1 cells (Supplementary Fig. S2B), suggesting that, through the postfix method, J073E5 detected mCCR3 specifically. By contrast, both C_3Mab-2 and recC_3Mab-2f did not bind to mCCR3 in CHO/mCCR3 using this method (Supplementary Fig. S2B). These results suggest that C_3Mab-2 and recC_3Mab-2f could not detect exogenous mCCR3 in an immunocytochemistry experiment.

Immunocytochemical analysis of P388 and J774-1 cells using C_3Mab-2 and recC_3Mab-2f

Next, we attempted to label endogenous mCCR3 with C_3Mab-2 (10 μg/mL) and recC_3Mab-2f (10 μg/mL) through the prefix method. Consequently, both antibodies recognized mCCR3 in the 4% PFA-fixed P388 and J774-1 cells (Fig. 1A, B). C_3Mab-2 and recC_3Mab-2f also detected endogenous mCCR3 in living P388 and J774-1 cells through the postfix method, suggesting that C_3Mab-2 and recC_3Mab-2f recognize the endogenous mCCR3 located on the cell surface (Fig. 2A, B). Although J073E5 showed nonspecific strong dot signals in P388 and J774-1 cells through the prefix method (Fig. 1A, B), it displayed specific but weak signals in both cell lines through the postfix method (Fig. 2A, B). These results suggest that C_3Mab-2 and recC_3Mab-2f can detect endogenous mCCR3 in immunocytochemistry experiments.

Discussion

This study demonstrated that C_3Mab-2 and recC_3Mab-2f could detect endogenous mCCR3 in the PFA-fixed P388 and J774-1 cells in immunocytochemistry experiments. Moreover, C_3Mab-2 and recC_3Mab-2f recognized endogenous mCCR3 in living P388 and J774-1 cells, indicating that both antibodies bind to the extracellular antigen region of the cell surface mCCR3. By contrast, C_3Mab-2 and recC_3Mab-2f did not allow the detection of exogenously expressed mCCR3 in CHO/mCCR3 cells. Although C_3Mab-2 detected mCCR3 in CHO/mCCR3 cells through flow cytometry, the fluorescence intensity was weaker than with J073E5.[17] We suppose that immunocytochemical methods are not sensitive enough to detect exogenous mCCR3 recognition by C_3Mab-2. However, we cannot exclude the possibility that C_3Mab-2 and recC_3Mab-2f can recognize exogenous mCCR3 expressed in other cell lines.

This study compared the immunolabeling of C_3Mab-2 and recC_3Mab-2f with that of J073E5, although J073E5 is only applicable to flow cytometry according to the manufacturer's technical datasheet. Regarding the strong nonspecific signals caused by J073E5 in PFA-fixed cells (prefix method) (Fig. 1 and Supplementary Fig. S1), we speculate that PFA altered the morphology of cell surface proteins, which interacted nonspecifically with J073E5. In this study, we can exclude an effect of residual PFA because ammonium chloride quenched it and because C_3Mab-2 and recC_3Mab-2f showed no nonspecific signal. By contrast, J073E5 specifically detected mCCR3 when we immunolabeled before fixing with PFA (postfix method) (Fig. 2 and Supplementary Fig. S2).

Some studies have demonstrated that antibodies against CCR3 or its ligands can be therapeutic agents against inflammatory diseases. Blocking CCR3 activity with CCR3 mAbs reduces eosinophilic inflammation, attenuating asthmatic response, gastroenteritis, and pulmonary inflammation.[18–20] An anti-CCL24 mAb suppresses the CCL24/CCR3 axis, repairing liver fibrosis and inflammation.[21] This study showed that C_3Mab-2 and recC_3Mab-2f react with endogenous mCCR3 in P388 and J774-1 cells, suggesting that both antibodies are usable for diagnosis and/or allergic inflammation and asthma medical treatment development in mouse models.

Moreover, CCR3 regulates tumor progression. CCL7 promotes the migration of oral squamous cell carcinoma cells through CCR1 and CCR3. Anti-CCR1 and anti-CCR3 mAbs block CCR1 and CCR3, suppressing migration.[22] The CCR3 inhibitor SB328437 inhibited clear-cell renal cell carcinoma progression through the autocrine-regulatory loop of gankyrin/STAT3/CCL24/CCR3 *in vitro* and *in vivo*.[23] Future studies should investigate (1) whether C_3Mab-2 and recC_3Mab-2f recognize endogenous CCR3 expressed by mouse cancer cells, (2) whether they suppress CCR3-mediated signal transduction, and (3) whether they show antitumor activity *in vitro* and *in vivo*.

In conclusion, our study reveals that C_3Mab-2 and recC_3Mab-2f are useful for detecting endogenous mCCR3 through immunocytochemistry experiments.

Author Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2

References

1. Korbecki J, Kojder K, Siminska D, Bohatyrewicz R, Gutowska I, Chlubek D, and Baranowska-Bosiacka I: CC chemokines in a tumor: A review of pro-cancer and anti-cancer properties of the ligands of receptors CCR1, CCR2, CCR3, and CCR4. Int J Mol Sci 2020;21:8412.
2. Gerber BO, Zanni MP, Uguccioni M, Loetscher M, Mackay CR, Pichler WJ, Yawalkar N, Baggiolini M, and Moser B: Functional expression of the eotaxin receptor CCR3 in T lymphocytes co-localizing with eosinophils. Curr Biol 1997;7:836–843.
3. Joubert P, Lajoie-Kadoch S, Labonte I, Gounni AS, Maghni K, Wellemans V, Chakir J, Laviolette M, Hamid Q, and Lamkhioued B: CCR3 expression and function in asthmatic airway smooth muscle cells. J Immunol 2005;175:2702–2708.

4. Ponath PD, Qin S, Post TW, Wang J, Wu L, Gerard NP, Newman W, Gerard C, and Mackay CR: Molecular cloning and characterization of a human eotaxin receptor expressed selectively on eosinophils. J Exp Med 1996;183:2437–2448.
5. Uguccioni M, Mackay CR, Ochensberger B, Loetscher P, Rhis S, LaRosa GJ, Rao P, Ponath PD, Baggiolini M, and Dahinden CA: High expression of the chemokine receptor CCR3 in human blood basophils. Role in activation by eotaxin, MCP-4, and other chemokines. J Clin Invest 1997; 100:1137–1143.
6. Tian M, Chen L, Ma L, Wang D, Shao B, Wu J, Wu H, and Jin Y: Expression and prognostic significance of CCL11/CCR3 in glioblastoma. Oncotarget 2016;7:32617–32627.
7. Liu F, and Wu H: CC chemokine receptors in lung adenocarcinoma: The inflammation-related prognostic biomarkers and immunotherapeutic targets. J Inflamm Res 2021;14:267–285.
8. Johrer K, Zelle-Rieser C, Perathoner A, Moser P, Hager M, Ramoner R, Gander H, Holtl L, Bartsch G, Greil R, and Thurnher M: Up-regulation of functional chemokine receptor CCR3 in human renal cell carcinoma. Clin Cancer Res 2005;11:2459–2465.
9. Jung MY, Kim SH, Cho D, and Kim TS: Analysis of the expression profiles of cytokines and cytokine-related genes during the progression of breast cancer growth in mice. Oncol Rep 2009;22:1141–1147.
10. Hosono H, Asano T, Takei J, Sano M, Tanaka T, Kaneko MK, and Kato Y: Development of an anti-elephant podoplanin monoclonal antibody PMab-265 for flow cytometry. Monoclon Antib Immunodiagn Immunother 2021;40:141–145.
11. Tanaka T, Asano T, Sano M, Takei J, Hosono H, Nanamiya R, Nakamura T, Yanaka M, Harada H, Fukui M, Suzuki H, Uchida K, Nakagawa T, Kato Y, and Kaneko MK: Development of monoclonal antibody PMab-269 against California sea lion podoplanin. Monoclon Antib Immunodiagn Immunother 2021;40:124–133.
12. Itai S, Fujii Y, Nakamura T, Chang YW, Yanaka M, Saidoh N, Handa S, Suzuki H, Harada H, Yamada S, Kaneko MK, and Kato Y: Establishment of CMab-43, a sensitive and specific anti-CD133 monoclonal antibody, for immunohistochemistry. Monoclon Antib Immunodiagn Immunother 2017;36:231–235.
13. Hosono H, Ohishi T, Takei J, Asano T, Sayama Y, Kawada M, Kaneko MK, and Kato Y: The anti-epithelial cell adhesion molecule (EpCAM) monoclonal antibody EpMab-16 exerts antitumor activity in a mouse model of colorectal adenocarcinoma. Oncol Lett 2020;20:383.
14. Yamada S, Itai S, Nakamura T, Yanaka M, Chang YW, Suzuki H, Kaneko MK, and Kato Y: Monoclonal antibody L_1Mab-13 detected human PD-L1 in lung cancers. Monoclon Antib Immunodiagn Immunother 2018;37:110–115.
15. Tanaka T, Nanamiya R, Takei J, Nakamura T, Yanaka M, Hosono H, Sano M, Asano T, Kaneko MK, and Kato Y: Development of anti-mouse CC chemokine receptor 8 monoclonal antibodies for flow cytometry. Monoclon Antib Immunodiagn Immunother 2021;40:65–70.
16. Nanamiya R, Takei J, Asano T, Tanaka T, Sano M, Nakamura T, Yanaka M, Hosono H, Kaneko MK, and Kato Y: Development of anti-human CC chemokine receptor 9 monoclonal antibodies for flow cytometry. Monoclon Antib Immunodiagn Immunother 2021;40:101–106.
17. Asano T, Nanamiya R, Takei J, Nakamura T, Yanaka M, Hosono H, Tanaka T, Sano M, Kaneko MK, and Kato Y: Development of anti-mouse CC chemokine receptor 3 monoclonal antibodies for flow cytometry. Monoclon Antib Immunodiagn Immunother 2021;40:107–112.
18. Song DJ, Shim MH, Lee N, Yoo Y, and Choung JT: CCR3 monoclonal antibody inhibits eosinophilic inflammation and mucosal injury in a mouse model of eosinophilic gastroenteritis. Allergy Asthma Immunol Res 2017;9:360–367.
19. Shen HH, Xu F, Zhang GS, Wang SB, and Xu WH: CCR3 monoclonal antibody inhibits airway eosinophilic inflammation and mucus overproduction in a mouse model of asthma. Acta Pharmacol Sin 2006;27:1594–1599.
20. Wang K, Shen HH, Li W, and Huang HQ: Human C-C chemokine receptor 3 monoclonal antibody inhibits pulmonary inflammation in allergic mice. Acta Pharmacol Sin 2007;28:1791–1796.
21. Segal-Salto M, Barashi N, Katav A, Edelshtein V, Aharon A, Hashmueli S, George J, Maor Y, Pinzani M, Haberman D, Hall A, Friedman S, and Mor A: A blocking monoclonal antibody to CCL24 alleviates liver fibrosis and inflammation in experimental models of liver damage. JHEP Rep 2020;2:100064.
22. Jung DW, Che ZM, Kim J, Kim K, Kim KY, Williams D, and Kim J: Tumor-stromal crosstalk in invasion of oral squamous cell carcinoma: A pivotal role of CCL7. Int J Cancer 2010;127:332–344.
23. Wang C, Wang Y, Hong T, Cheng B, Gan S, Chen L, Zhang J, Zuo L, Li J, and Cui X: Blocking the autocrine regulatory loop of Gankyrin/STAT3/CCL24/CCR3 impairs the progression and pazopanib resistance of clear cell renal cell carcinoma. Cell Death Dis 2020;11:117.

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