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**Critical involvement of atypical chemokine receptor CXCR7 in allergic airway inflammation**

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## **Abstract**

Trafficking and recruitment of immune cells to the site of inflammation with spatial and temporal synchronization is crucial for the development of allergic airway inflammation. Particularly, chemokines are known to be key players in these processes. Previous studies revealed that the CXCL12/CXCR4 axis plays an important role in regulating allergic airway inflammation. However, the role of CXCR7, a recently discovered second receptor for CXCL12, in regulating airway inflammation has not been explored. Initially, CXCR7 was considered as a decoy receptor; however, numerous subsequent studies revealed that engagement of CXCR7 triggered its own signaling or modulated CXCR4-mediated signaling. In the present study, we detected the expression of CXCR7 in airway epithelial cells. Use of a lentiviral-delivery system to knock down the expression of CXCR7 in the lung of sensitized mice abrogated the cardinal features of asthma, indicating that CXCR7 plays a role in regulating allergic airway inflammation. The activation of MAPK and AKT signaling in response to CXCL12 in mouse epithelial cell line, MLE-12, was reduced when CXCR7 expression was knocked down. However, either knockdown or overexpression of CXCR7 in MLE-12 did not

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affect CXCL12-mediated calcium influx, indicating that CXCR7 does not modulate CXCR4-mediated signaling, and that it functions as a signaling receptor rather than a decoy receptor. Finally, we found that the expression of chemokine CCL2 is regulated by CXCR7/CXCL12-mediated signaling through  $\beta$ -arrestin in airway epithelial cells. Thus, regulating the expression of CCL2 in airway epithelial cells may be one mechanism by which CXCR7 participates in regulating allergic airway inflammation.

## Introduction

Allergic airway inflammation is a disorder in which sensitized individuals develop eosinophilic airway inflammation, mucus hyper-secretion, and airway hyperresponsiveness in response to inhaled aeroallergens. These features are orchestrated by dendritic cells, T<sub>H</sub>2 cells, eosinophils, mast cells, and airway structure cells, such as epithelial cells. Trafficking and recruitment of leukocytes with spatial and temporal synchronization is important for establishing allergic airway inflammation. Chemokines, small chemotactic proteins, are key players in these processes and allow leukocytes with a corresponding receptor to migrate from the bloodstream into interstitial spaces and contribute to the inflammation process.

CXCL12, also known as stromal-derived factor-1, was originally identified as a pre-B cell growth factor<sup>1</sup>. Despite its roles in hematopoiesis and inflammation, CXCL12 also has important functions in various biological processes, including cardiac and neuronal development, stem cell

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motility, neovascularization, angiogenesis, apoptosis, tumorigenesis, and metastasis <sup>2-6</sup>. CXCR4 has long been thought to be the only receptor for CXCL12, as CXCR4- and CXCL12-deficient mice have similar phenotypes <sup>7, 8</sup>. Evidence from human and animal studies revealed that CXCL12/CXCR4 plays an important role in allergic airway inflammation <sup>9-12</sup>. Using a CXCR4 or CXCL12 neutralization antibody or the CXCR4 antagonist AMD3100, the CXCL12/CXCR4 axis has been demonstrated to play a pivotal role in airway inflammation and airway hyperresponsiveness in an allergic airway-inflammation mouse model <sup>9, 10</sup>. Indeed, in patients with asthma, a high concentration of CXCL12 was observed in the bronchoalveolar lavage fluid (BALF), and levels of the chemokine were correlated with the number of leukocytes, including lymphocytes, macrophages, and eosinophils, indicating that CXCL12 contributes to their recruitment into the airway <sup>11</sup>.

Recently, the orphan receptor RDC-1, now known as CXCR7, was demonstrated to be an additional receptor for CXCL12 <sup>13, 14</sup>. Its binding affinity to CXCL12 is 5–10-fold higher than that of CXCR4 to CXCL12 <sup>13, 15</sup>. It is well accepted that CXCR7 is an atypical chemokine receptor, as engagement of CXCR7 does not trigger typical chemokine receptor signaling, such as calcium mobilization and chemotaxis <sup>14, 16</sup>. Therefore, CXCR7 has been suggested to function as a decoy receptor that sequesters/scavenges CXCL12 from the environment, thereby shaping the availability of the chemokine for the signaling receptor CXCR4 <sup>17-21</sup>. However, studies of different cells and pathophysiological conditions have revealed a broad range of CXCR7 functions, showing that CXCL12 engagement to CXCR7 can induce a range of cellular responses, such as activation of the

MAPK and AKT pathways<sup>22</sup>, receptor internalization<sup>13, 17</sup>, cell survival<sup>14, 22-24</sup>, proliferation<sup>25</sup>, and adhesion<sup>22, 23, 26</sup>. Indeed, overexpression studies showed that when heterodimerized with CXCR4, CXCR7 can influence CXCR4-dependent G protein-mediated cell signaling, therefore affecting the role of CXCR4 in cellular physiology<sup>16, 26-28</sup>. Recently, other studies showed that  $\beta$ -arrestin-2, other than G protein-mediated signaling, is triggered by the engagement of CXCR7<sup>27, 29-31</sup>.

As the second and newly identified receptor for CXCL12, the role of CXCR7 in allergic airway inflammation has not been evaluated. In the present study, we examined whether CXCR7 is involved in regulating allergic airway inflammation. Our results suggest that epithelial-CXCR7 may be involved in regulating cardinal features of asthma by functioning as a signaling receptor rather than a decoy receptor, without affecting CXCR4-mediated signaling. One of the possible mechanisms by which epithelial-CXCR7 regulate allergic airway inflammation is through regulating the expression of CCL2 during inflammation.

## **Materials and methods**

### **Animals and cells:**

Female BALB/c mice (6–8-week old) were purchased from the National Laboratory Animal Center of Taiwan. All experiments conducted in this study were in accordance with the Institutional Animal Care and Use Committee of China Medical University according to Care of the animals and surgical

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procedures of China Medical University Protocols (Approval protocol number: 102-183-N). MLE-12, a mouse respiratory epithelial cell line obtained from ATCC (Manassas, VA, USA), was cultured in DMEM containing 10% fetal bovine serum.

#### **Immunofluorescence staining of CXCR7 and CXCR4**

Lungs were inflated with a mixture of 1:1 OCT/PBS and snap-frozen in OCT on dry ice. Frozen sections (10  $\mu$ m) were prepared, fixed with cold acetone for 3 min, dried, and washed. The tissue sections were blocked using 1% bovine serum albumin (BSA) in PBST (PBS containing 0.05% Tween-20) and stained with CXCR7 (11G8) (R&D Systems, Minneapolis, MN, USA) or CXCR4 (H-118) (Santa Cruz Biotechnology, Dallas, TX, USA) antibodies. The tissue sections were then incubated with secondary antibodies, Alexa Fluor 488–conjugated chicken anti-rabbit IgG and Alexa Fluor 594–conjugated chicken anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA, USA), respectively. Nuclei were counterstained with DAPI (Sigma-Aldrich, St. Louis, MO, USA). Protein expression was observed using a fluorescence microscope ECLIPSE 80i (Nikon Instruments, Inc., Tokyo, Japan).

### **Murine model of allergic airway inflammation**

The protocol has been described previously <sup>32</sup>. Briefly, the scheme of animal sensitization, treatment, and challenge is shown in Figure 1. Cytospin preparations from bronchoalveolar lavage fluid (BALF), obtained by flushing the lungs with PBS, were stained with Pappenheim stain for cell differential analysis. Serum obtained by cardiac puncturing was used to measure ovalbumin (OVA)-specific IgE. Cells from mediastinal lymph nodes were prepared for the T cell re-stimulation assay <sup>32</sup>. The concentrations of cytokines in culture supernatants after 48 h of culture were measured using a Duo Set ELISA kit (R&D Systems). Whole lung lobes were dissected for histological and biochemical analyses.

### **Assay of airway hyperresponsiveness**

Airway resistance was assessed by invasive body plethysmography (flexiVent, SCIPEQ Scientific Respiratory Equipment Inc., Montreal, Quebec, Canada). The detailed protocol was described previously <sup>32</sup>.

### **Western blot analysis**

Total cellular protein was prepared by lysing cells with SDS sample buffer. The proteins were separated on a 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. Membranes were blocked with PBS containing 5% BSA and incubated with the indicated antibodies. The blots were further incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies and developed using an enhanced chemiluminescence detection system (PerkinElmer, Waltham, MA, USA) according to the manufacturer's instructions. The intensity of each band was quantified and analyzed using Image J software (Bethesda, MD, USA).

### **Lentivirus preparation and stable silencing of target genes**

Lentivirus bearing target gene shRNA was prepared according the protocols provided by the National Core Facility for Manipulation of Gene Function by RNAi, miRNA, miRNA sponges, and CRISPR/Genomic Research Center at Academia Sinica in Taiwan.

The target sequences for mouse CXCR7 were:

shRNA#1: 5'-GCCTGGCAACTACTCTGACAT-3' (TRCN0000026660)

shRNA#2: 5'-GCCTTCATCTTCAAGTACTCG-3'<sup>24</sup>.

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The target sequences for mouse  $\beta$ -arrestin2 were:

5'-CCTCATCGAATTCGATACCAA-3' (TRCN0000287639)

MLE-12 cells ( $5 \times 10^5$ ) were transfected with lentiviral expression vector containing shRNA in 6-well culture plates and selected in medium containing 0.75  $\mu$ g/mL puromycin 1 day later for stable silencing of the target gene.

#### **Calcium mobilization assay**

Calcium mobilization responses were measured using Fluo3 according to the manufacturer's instructions (Invitrogen). MLE-12 WT cells were loaded with 10 nM/mL Fluo3 and 0.02% F127 for 30 min at 37°C in a 5% CO<sub>2</sub> incubator. The cells were then treated with or without 250 ng/mL CXCL-12 (R&D Systems). Fluo-3 was excited at 488 nm and emitted fluorescence measured with a 515-nm long pass filter. The relative fluorescence unit (RFU) of calcium binding was measured using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

## **Statistical analysis**

The results were compared using one-way analysis of variance (ANOVA) with the software program GraphPad Prism 5 (GraphPad, Inc., La Jolla, CA, USA). The data are presented as the mean  $\pm$  standard error of mean (SEM). Probability values (p) of less than 0.05 were considered statistically significant.

## **Results**

### **Identification of CXCR7 expression in airway epithelial cells**

To examine whether CXCR7 is expressed in the lung, western blotting analysis was performed using the protein prepared from lungs of naïve mice or allergen-induced airway-inflamed mice. As shown in Figure 2A, the expression of CXCR7 was increased in the lungs of inflamed mice. Furthermore, immunofluorescence staining of lung tissue from inflamed mice indicated that CXCR7 was predominately expressed in epithelial cells. In contrast to CXCR4, which was expressed in airway epithelial cells, the expression of CXCR7 can be further detected in vessel endothelial cells (Figure 2B).

## Knockdown of CXCR7 in the lung reduced allergic airway inflammation

Because CXCR7 knockout mice show early postnatal lethality<sup>16,33</sup>, to examine whether CXCR7 plays a role in regulating T<sub>H</sub>2-mediated allergic airway inflammation, we carried out an experiment using a murine asthma model in which CXCR7 was knocked-down by intratracheally delivering lentivirus carrying CXCR7 shRNA into sensitized mice before challenge (Figure 1). To investigate the effect of CXCR7 on airway inflammation, total and differential cell counts in BALF were analyzed after the mice were challenged with the allergen OVA. The relative expression levels of CXCR7 in the lung of challenged mice are shown in Supplemental Fig. S1. When CXCR7 expression was knocked down before challenge, less cell infiltration was observed in the lungs of challenged mice (Figure 3A). Histological examination of fixed lung tissue prepared from these mice revealed that the extent of peribronchiolar cell inflammation and mucus secretion was lower in mice that received lentivirus carrying CXCR7 shRNA than in mice that received lentivirus carrying the control vector alone (Figure 3B).

## **Knockdown of CXCR7 in the lung reduced serum allergen-specific IgE production and T cell cytokine production**

Elevated levels of IgE in patients with asthma are also an indicator of allergy. Therefore, we examined whether knockdown of CXCR7 in the lung could reduce the level of OVA-specific IgE in the serum. We found that OVA-specific IgE levels were significantly reduced (Figure 4A).

T<sub>H</sub>2 cells plays a central role in the disease process, contributing to IgE production by B cells, growth and differentiation of eosinophils and mast cells, and the development of bronchial hyperactivity and goblet cell hyperplasia. Thus, to further establish whether the T cell response was defective, cytokine production by mediastinal LN (mLN) cells isolated from mice following restimulation with antigen presenting cells and OVA were analyzed by enzyme-linked immunosorbent assay (ELISA). As shown in Figure 4B and Supplemental Figure S2, T<sub>H</sub>2-related cytokine production was diminished when CXCR7 expression was knocked down in the lung. Because CXCR7 was knocked-down after sensitization, the function and numbers of existing effector and memory T cells were the same as in the control, indicating that the re-activation/expansion of effector/memory cells was defective during challenge when CXCR7 expression was knocked down.

## **Development of airway hyperresponsiveness was reduced when CXCR7 was knocked down in the lung**

We subsequently examined whether knockdown of CXCR7 expression in the lung suppressed the development of airway hyperresponsiveness. As shown in Figure 5, CXCR7-knockdown mice showed significant decreases in pulmonary resistance compared to the control.

## **CXCR7 functions as a signaling receptor rather than a decoy receptor in lung epithelial cells**

Depending on the cell type, tissue, and pathophysiological state, CXCR7 can function as a decoy receptor or signaling receptor. As shown in Figure 2B, both CXCR7 and CXCR4 were expressed in airway epithelial cells; thus, it is unlikely that CXCR7 functions as a decoy receptor that sequesters CXCR12 from the environment to shape the availability for the signaling receptor CXCR4. As we delivered lentivirus carrying CXCR7 shRNA into mice intratracheally, the cell types infected were predominately epithelial cells. To confirm whether engagement of CXCR7 participates in CXCL12-mediated lung epithelial signaling, MLE-12 cells, a mouse lung epithelial cell line, and their derived control (bearing control vector), as well as a CXCR7-knockdown cell line (bearing CXCR7 shRNA expression construct) were stimulated with CXCL12, after which the activation of MAPK (ERK, JNK, and P38) and AKT were analyzed. Knockdown efficacy is shown in Supplemental Fig. S3. While CXCL12 activated MAPK and AKT signaling in wild-type and control

MLE-12 cells, the knockdown of CXCR7 attenuated the intensity of the CXCL12-MAPK and CXCL12-AKT signals (Figure 6A and Supplemental Figure S4), indicating that CXCR7 participates in CXCL-12-mediated signaling.

Although, the engagement of CXCR7 does not trigger typical GPCR-dependent signaling, it has been proposed that CXCR7 might serve as a co-receptor for CXCR4 and influence CXCL12/CXCR4-mediated G-protein signaling<sup>16, 26, 28</sup>. CXCR7 knockdown does not alter the expression of CXCR4 in airway epithelial cells (Fig. S3), however, to clarify whether CXCR7 might influence CXCL12/CXCR4-mediated G-protein signaling, calcium influx, a typical *Gai*-coupling-mediated signaling, induced by CXCL12 was examined. No significant difference was observed between wild-type airway epithelial cells and CXCR7-knockdown cells (Figure 6B) or cells overexpressing CXCR7 (Figure 6C), indicating that engagement of CXCR7 by CXCL12 in airway epithelial cells triggers signaling and does not interfere with CXCR4/CXCL-12-mediated signaling.

#### **CXCR7 signal elevates the expression levels of CCL2 in airway epithelial cells**

To identify the potential mediators induced by CXCR7 engagement that participate in regulating allergic airway inflammation, a cytokine protein array was used to examine the cytokine profile in the BALF of challenged mice. We found that the concentration of monocyte chemotactic protein-1 (MCP-1), also known as CCL2, was also reduced (Figure 7A). CCL2 can be produced by lung

epithelial cells and monocytes (51) and is known to recruit CCR2<sup>+</sup> monocytes to the site of inflammation and plays an important role in asthmatic responses<sup>34-37</sup>. To confirm that airway epithelial cells can produce CCL2 in response to CXCL-12, MLE-12 was stimulated with CXCL-12. As shown in Fig 7B, CXCL-12 can induce airway epithelial cell to produce CCL2. To confirm that airway epithelial cells can produce CCL2 through the CXCL12/CXCR7-mediated pathway, MLE-12 was stimulated with CXCL-12. Indeed, when CXCR7 was overexpressed in airway epithelial cells, the production of CCL2 by these cells was augmented (Figure 7C). This indicates that CXCR7 could regulate the production of CCL2 in airway epithelial cells. To examine whether CXCL12/CXCR7-induced CCL2 expression bypasses the CXCL12/CXCR4-mediated signaling pathway, AMD3100, a CXCR4 antagonist was used. As shown in Fig 7B, in response to CXCL12, the production of CCL2 was not altered when CXCR4-mediated signaling was blocked by AMD3100, indicating that CXCL12 induced expression of CCL2 is not through CXCR4. It is known that  $\beta$ -arrestin-2, other than G protein-mediated signaling, is triggered by the engagement of CXCR7<sup>27, 29-31</sup>. To examine whether CXCL12/CXCR7-induced CCL2 expression is through  $\beta$ -arrestin,  $\beta$ -arrestin was knocked down in MLE-12 cells and the production of CCL2 in response to CXCL12 was examined. As shown in Fig 7D, the production of CCL2 was decreased when  $\beta$ -arrestin was knocked down in MLE-12 cells. These data indicate that CXCL12/CXCR7 induced CCL2 expression through  $\beta$ -arrestin, without bypassing the CXCL12/CXCR4-mediated signaling pathway.

## Discussion

Our data indicate that CXCR7 functions as a signaling receptor and is expressed in the airway epithelium. Its expression was further augmented under inflamed conditions. Engagement of CXCR7 is involved in regulating the cardinal features of asthma, as knockdown of CXCR7 in the lung of sensitized mice using a lentiviral-delivery system abrogated the characteristic features of asthma, such as inflammation, mucus secretion, serum IgE concentration, and airway hyperresponsiveness. We further found that these defective features may have resulted in part from lower production of CCL2 in the lung.

CCL2 (monocyte chemotactic protein, MCP-1/CCL2) is expressed by many different cell types, including epithelial cells. CCL2 is a potent chemoattractant not only for macrophages, but also for basophils and activated and memory T cells<sup>38-41</sup>. Several studies have suggested that CCL2/CCR2 play important roles in the pathogenesis of asthma<sup>42</sup>. Elevated CCL2 levels are present in the BALF of asthmatic individuals<sup>37</sup>. Significant release of CCL2 into the airway after endobronchial challenge has also been observed in asthmatic patients<sup>36</sup>. Polymorphisms in the CCL2 gene are associated with asthma in children and adult<sup>43</sup>. When CCL2 activity was blocked with neutralization antibodies, the levels of airway inflammation and hyperresponsiveness were attenuated<sup>34, 35, 42</sup>. Additionally, in CCR2-deficient mice, airway hyperresponsiveness were significantly reduced<sup>34</sup>. Furthermore, CCL2 was also shown to influence T cell differentiation towards T<sub>H</sub>2 cells and enhance T cells to produce IL-4<sup>44, 45</sup>.

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In this study, we observed defective recall T cell responses in the lymph node (Figure 4B and Figure 1S). Because CXCR7 was knocked-down before challenge, the function and numbers of existing effector and memory T cells before challenge were expected to be the same between control and experimental groups. It is likely that the re-activation/expansion of effector/memory cells was defective, resulting in a reduced number of effector T cells in the mLN, and thus fewer activated effector T cells in the lymph node were available for restimulation.

Dendritic cells (DCs) are known to be important in inducing T helper cell immunity in the lymph node <sup>46,47</sup>. When CD11c<sup>+</sup> cells were conditionally depleted locally from the lung immediately prior to the antigen (OVA) challenge in OVA-sensitized mice, van Rijt LS et al. observed that in the absence of DCs, the cardinal features of asthma failed to develop and that *ex vivo* isolated CD4 T cells produced greatly diminished amounts of T<sub>H</sub>2-related cytokines <sup>46</sup>, indicating that DCs are key cells in the development of secondary immune responses. DCs in the lung are comprised of heterogeneous populations including conventional CD11b<sup>+</sup> DCs, CD103<sup>+</sup> DCs, plasmacytoid DCs, and monocyte-derived DCs (moDCs). Among these cells, only conventional CD11b<sup>+</sup> CD64<sup>+</sup>MAR-1<sup>-</sup>DCs and CD11b<sup>+</sup> CD64<sup>+</sup>MAR-1<sup>+</sup> moDCs are principle subsets for inducing T<sub>H</sub>2 cell-mediated immunity <sup>47</sup>. Upon inhalation of an antigen, lung epithelial cells produce CCL2 and CCL7, which induce the release of CCR2<sup>+</sup> monocytes from the bone marrow. When the monocytes reach the airway, they give rise to not only monocyte-derived macrophages, but also monocyte-derived DCs. Interestingly, we found that the concentrations of CCL2 in the BALF of CXCR7-knockdown inflamed mice were

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reduced, which may explain why we observed fewer macrophages in the BALF (Figure. 3A). We further demonstrated that CCL2 expression was regulated by CXCL12/CXCR7-mediated signaling in lung epithelial cells. Epithelial cell-derived products clearly have the potential to modulate the lung microenvironment and DC functional maturation. Thus, one of the mechanisms by which CXCR7 regulates allergic airway inflammation may be through regulating the expression of epithelial cell-derived proteins such as CCL2, which may influence DC functional maturation or/and regulate the recruitment of monocytes. This subsequently gives rise to DCs, thus increasing the number of antigen-bearing DCs migrating to the draining lymph node. Thus, it would be of interest to examine the function and number of DCs that migrate into mediastinal lymph nodes.

In conclusion, the present study demonstrated that expression of CXCR7 in the lung is upregulated during inflamed conditions and that engagement of CXCR7 with CXCL12 triggers signal transduction and regulates gene expressions. CCL2, an important mediator of allergic immune responses, is one of the molecules regulated by CXCR7-mediated signaling, implicating the critical role of CXCR7 in regulating allergic airway inflammation. Thus, CXCR7 could be a potential therapeutic target for allergic airway diseases.

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## **Conflict of interest**

The authors declare no conflict of interest

## References

1. Nagasawa T, Kikutani H, Kishimoto T. Molecular cloning and structure of a pre-B-cell growth-stimulating factor. *Proc Natl Acad Sci U S A* 1994;91(6):2305-9.
2. Atluri P, Woo YJ. Pro-angiogenic cytokines as cardiovascular therapeutics: assessing the potential. *BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy* 2008;22(4):209-22.
3. Chen M, Xie HQ, Deng L, Li XQ, Wang Y, Zhi W, et al. Stromal cell-derived factor-1 promotes bone marrow-derived cells differentiation to cardiomyocyte phenotypes in vitro. *Cell proliferation* 2008;41(2):336-47.
4. Hernandez-Lopez C, Valencia J, Hidalgo L, Martinez VG, Zapata AG, Sacedon R, et al. CXCL12/CXCR4 signaling promotes human thymic dendritic cell survival regulating the Bcl-2/Bax ratio. *Immunol Lett* 2008;120(1-2):72-8.
5. Schonemeier B, Kolodziej A, Schulz S, Jacobs S, Hoell V, Stumm R. Regional and cellular localization of the CXCL12/SDF-1 chemokine receptor CXCR7 in the developing and adult rat brain. *J Comp Neurol* 2008;510(2):207-20.
6. Zhang S, Qi L, Li M, Zhang D, Xu S, Wang N, et al. Chemokine CXCL12 and its receptor CXCR4 expression are associated with perineural invasion of prostate cancer. *J Exp Clin Cancer Res* 2008;27:62.

- Accepted Article
7. Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* 1998;393(6685):595-9.
  8. Ma Q, Jones D, Borghesani PR, Segal RA, Nagasawa T, Kishimoto T, et al. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc Natl Acad Sci U S A* 1998;95(16):9448-53.
  9. Gonzalo JA, Lloyd CM, Peled A, Delaney T, Coyle AJ, Gutierrez-Ramos JC. Critical involvement of the chemotactic axis CXCR4/stromal cell-derived factor-1 alpha in the inflammatory component of allergic airway disease. *J Immunol* 2000;165(1):499-508.
  10. Lukacs NW, Berlin A, Schols D, Skerlj RT, Bridger GJ. AMD3100, a CxCR4 antagonist, attenuates allergic lung inflammation and airway hyperreactivity. *The American journal of pathology* 2002;160(4):1353-60.
  11. Negrete-Garcia MC, Velazquez JR, Popoca-Coyotl A, Montes-Vizuet AR, Juarez-Carvajal E, Teran LM. Chemokine (C-X-C motif) ligand 12/stromal cell-derived factor-1 is associated with leukocyte recruitment in asthma. *Chest* 2010;138(1):100-6.
  12. Hachet-Haas M, Balabanian K, Rohmer F, Pons F, Franchet C, Lecat S, et al. Small neutralizing molecules to inhibit actions of the chemokine CXCL12. *J Biol Chem* 2008;283(34):23189-99.

- Accepted Article
13. Balabanian K, Lagane B, Infantino S, Chow KY, Harriague J, Moepps B, et al. The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *J Biol Chem* 2005;280(42):35760-6.
  14. Burns JM, Summers BC, Wang Y, Melikian A, Berahovich R, Miao Z, et al. A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *J Exp Med* 2006;203(9):2201-13.
  15. Crump MP, Gong JH, Loetscher P, Rajarathnam K, Amara A, Arenzana-Seisdedos F, et al. Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. *EMBO J* 1997;16(23):6996-7007.
  16. Sierro F, Biben C, Martinez-Munoz L, Mellado M, Ransohoff RM, Li M, et al. Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proc Natl Acad Sci U S A* 2007;104(37):14759-64.
  17. Boldajipour B, Mahabaleshwar H, Kardash E, Reichman-Fried M, Blaser H, Minina S, et al. Control of chemokine-guided cell migration by ligand sequestration. *Cell* 2008;132(3):463-73.
  18. Cruz-Orengo L, Holman DW, Dorsey D, Zhou L, Zhang P, Wright M, et al. CXCR7 influences leukocyte entry into the CNS parenchyma by controlling abluminal CXCL12 abundance during autoimmunity. *The Journal of experimental medicine* 2011;208(2):327-39.

- Accepted Article
19. Sanchez-Alcaniz JA, Haegel S, Mueller W, Pla R, Mackay F, Schulz S, et al. Cxcr7 controls neuronal migration by regulating chemokine responsiveness. *Neuron* 2011;69(1):77-90.
  20. Wang Y, Li G, Stanco A, Long JE, Crawford D, Potter GB, et al. CXCR4 and CXCR7 have distinct functions in regulating interneuron migration. *Neuron* 2011;69(1):61-76.
  21. Dambly-Chaudiere C, Cubedo N, Ghysen A. Control of cell migration in the development of the posterior lateral line: antagonistic interactions between the chemokine receptors CXCR4 and CXCR7/RDC1. *BMC Dev Biol* 2007;7:23.
  22. Wang J, Shiozawa Y, Wang Y, Jung Y, Pienta KJ, Mehra R, et al. The role of CXCR7/RDC1 as a chemokine receptor for CXCL12/SDF-1 in prostate cancer. *The Journal of biological chemistry* 2008;283(7):4283-94.
  23. Mazzinghi B, Ronconi E, Lazzeri E, Sagrinati C, Ballerini L, Angelotti ML, et al. Essential but differential role for CXCR4 and CXCR7 in the therapeutic homing of human renal progenitor cells. *The Journal of experimental medicine* 2008;205(2):479-90.
  24. Miao Z, Luker KE, Summers BC, Berahovich R, Bhojani MS, Rehemtulla A, et al. CXCR7 (RDC1) promotes breast and lung tumor growth in vivo and is expressed on tumor-associated vasculature. *Proc Natl Acad Sci U S A* 2007;104(40):15735-40.
  25. Meijer J, Ogink J, Roos E. Effect of the chemokine receptor CXCR7 on proliferation of carcinoma cells in vitro and in vivo. *Br J Cancer* 2008;99(9):1493-501.

26. Hartmann TN, Grabovsky V, Pasvolsky R, Shulman Z, Buss EC, Spiegel A, et al. A crosstalk between intracellular CXCR7 and CXCR4 involved in rapid CXCL12-triggered integrin activation but not in chemokine-triggered motility of human T lymphocytes and CD34+ cells. *J Leukoc Biol* 2008;84(4):1130-40.
27. Decaillot FM, Kazmi MA, Lin Y, Ray-Saha S, Sakmar TP, Sachdev P. CXCR7/CXCR4 heterodimer constitutively recruits beta-arrestin to enhance cell migration. *The Journal of biological chemistry* 2011;286(37):32188-97.
28. Levoe A, Balabanian K, Baleux F, Bachelier F, Lagane B. CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. *Blood* 2009;113(24):6085-93.
29. Rajagopal S, Kim J, Ahn S, Craig S, Lam CM, Gerard NP, et al. Beta-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7. *Proc Natl Acad Sci U S A* 2010;107(2):628-32.
30. Luker KE, Gupta M, Steele JM, Foerster BR, Luker GD. Imaging ligand-dependent activation of CXCR7. *Neoplasia* 2009;11(10):1022-35.
31. Reiter E, Ahn S, Shukla AK, Lefkowitz RJ. Molecular Mechanism of  $\beta$ -Arrestin-Biased Agonism at Seven-Transmembrane Receptors. *Annual review of pharmacology and toxicology* 2011.

- Accepted Article
32. Chen JH, Huang PH, Lee CC, Chen PY, Chen HC. A bovine whey protein extract can induce the generation of regulatory T cells and shows potential to alleviate asthma symptoms in a murine asthma model. *Br J Nutr* 2013;109(10):1813-20.
33. Gerrits H, van Ingen Schenau DS, Bakker NE, van Disseldorp AJ, Strik A, Hermens LS, et al. Early postnatal lethality and cardiovascular defects in CXCR7-deficient mice. *Genesis* 2008;46(5):235-45.
34. Campbell EM, Charo IF, Kunkel SL, Strieter RM, Boring L, Gosling J, et al. Monocyte chemoattractant protein-1 mediates cockroach allergen-induced bronchial hyperreactivity in normal but not CCR2<sup>-/-</sup> mice: the role of mast cells. *J Immunol* 1999;163(4):2160-7.
35. Gonzalo JA, Lloyd CM, Wen D, Albar JP, Wells TN, Proudfoot A, et al. The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. *J Exp Med* 1998;188(1):157-67.
36. Holgate ST, Bodey KS, Janezic A, Frew AJ, Kaplan AP, Teran LM. Release of RANTES, MIP-1 alpha, and MCP-1 into asthmatic airways following endobronchial allergen challenge. *Am J Respir Crit Care Med* 1997;156(5):1377-83.
37. Sousa AR, Lane SJ, Nakhosteen JA, Yoshimura T, Lee TH, Poston RN. Increased expression of the monocyte chemoattractant protein-1 in bronchial tissue from asthmatic subjects. *Am J Respir Cell Mol Biol* 1994;10(2):142-7.

- Accepted Article
38. Conti P, Boucher W, Letourneau R, Feliciani C, Reale M, Barbacane RC, et al. Monocyte chemotactic protein-1 provokes mast cell aggregation and [3H]5HT release. *Immunology* 1995;86(3):434-40.
39. Bischoff SC, Krieger M, Brunner T, Dahinden CA. Monocyte chemotactic protein 1 is a potent activator of human basophils. *J Exp Med* 1992;175(5):1271-5.
40. Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, et al. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med* 1998;187(1):129-34.
41. Sallusto F, Lenig D, Mackay CR, Lanzavecchia A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J Exp Med* 1998;187(6):875-83.
42. Lukacs NW, Strieter RM, Warmington K, Lincoln P, Chensue SW, Kunkel SL. Differential recruitment of leukocyte populations and alteration of airway hyperreactivity by C-C family chemokines in allergic airway inflammation. *J Immunol* 1997;158(9):4398-404.
43. Szalai C, Kozma GT, Nagy A, Bojszko A, Krikovszky D, Szabo T, et al. Polymorphism in the gene regulatory region of MCP-1 is associated with asthma susceptibility and severity. *The Journal of allergy and clinical immunology* 2001;108(3):375-81.
44. Chensue SW, Warmington KS, Ruth JH, Sanghi PS, Lincoln P, Kunkel SL. Role of monocyte chemoattractant protein-1 (MCP-1) in Th1 (mycobacterial) and Th2 (schistosomal) antigen-induced
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granuloma formation: relationship to local inflammation, Th cell expression, and IL-12 production. J Immunol 1996;157(10):4602-8.

45. Karpus WJ, Lukacs NW, Kennedy KJ, Smith WS, Hurst SD, Barrett TA. Differential CC chemokine-induced enhancement of T helper cell cytokine production. J Immunol 1997;158(9):4129-36.

46. van Rijt LS, Jung S, Kleinjan A, Vos N, Willart M, Duez C, et al. In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. J Exp Med 2005;201(6):981-91.

47. Plantinga M, Guilliams M, Vanheerswynghe M, Deswarte K, Branco-Madeira F, Toussaint W, et al. Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. Immunity 2013;38(2):322-35.

## Figure Legends

**Figure 1. Brief scheme of animal sensitization, treatment, and challenge.** I.P: intra-peritoneal; I.T.: intra-tracheal; I.N.: intra-nasal; OVA: ovalbumin; Alum: alumini hydroxide.

**Figure 2. Expression of CXCR7 in the lung.** (A) The expression level of CXCR7 in the lung was analyzed by western blot analysis. The protein extract was prepared from the lungs of three naïve and three OVA-sensitized and challenged inflamed BALB/c mice. Band intensity was quantified and analyzed with Image J software after normalizing the expression levels of protein to  $\beta$ -actin. Numbers represent the mean  $\pm$  SEM of relative expression level to naïve-1. \*  $p < 0.001$  compared with naïve. Statistical significance was determined using Student's  $t$  test. (B) Frozen mouse lung sections derived from inflamed BALB/c mice were evaluated for the expression of CXCR7 (green) and CXCR4 (red) using immunofluorescence staining.

**Figure 3. Reduced airway inflammation following CXCR7 knockdown** (A) BAL fluid (BALF) total cell number (left) and differential cell counts (right) following CXCR7 knockdown virus delivery and three OVA-challenges were assessed by Pappenheim stain. Numbers represent the mean  $\pm$  SEM of cell number ( $n \geq 4$ ). \*  $p < 0.05$ ; \*\*\*  $p < 0.001$  compared with Lenti-Control. Statistical significance was determined using one-way ANOVA. Data shown are representative of three independent experiments. (B) Representative lung sections stained with hematoxylin and eosin (H&E; X200) or periodic acid-Schiff (PAS; X200). Arrows indicate areas of airway infiltrate (H&E) or presence of mucus substance (PAS). Numbers for H& E staining represent the mean  $\pm$  SEM of infiltrating inflammatory cells in subepithelial and subendothelial area ( $\text{mm}^2$ ) in lung section ( $n = 3$ ). Numbers for PAS staining represent the mean  $\pm$  SEM of H-Score ( $n = 3$ ). \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$

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compared with Lenti-Control. Statistical significance was determined by one-way ANOVA. **NC**: negative control, mice sensitized and challenged with PBS. **PC**: positive control, mice sensitized and challenged with OVA. **Lenti-Control**: lentivirus carrying control vector was intratracheally administered to sensitized mice 2 weeks before challenge. **Lenti-CXCR7 shRNA**: lentivirus carrying CXCR7 shRNA was intratracheally administered to sensitized mice 2 weeks before challenge.

**Figure 4. Serum OVA-specific IgE and cytokine production was reduced in CXCR7 knockdown mice.** (A) OVA-specific IgE in the serum was measured by ELISA. Numbers represent the mean  $\pm$  SEM of optical density (O.D.; n = 4). \*\*\* p < 0.001 compared with Lenti-Control. Statistical significance was determined by one-way ANOVA. (B) Cytokines production by mediastinal LN cells isolated from mice following re-stimulation with antigen presenting cells and OVA were analyzed by ELISA. In each experiment, mLN cells in the same group were pooled for culturing. Data shown is representative of three independent experiments. Data for an additional two additional experiments are shown in Supplemental Figure S2. **NC**: negative control, mice sensitized and challenged with PBS. **PC**: positive control, mice sensitized and challenged with OVA. **Lenti-Control**: lentivirus carrying control vector was intratracheally administered to sensitized mice 2 weeks before challenge. **Lenti-CXCR7 shRNA**: lentivirus carrying CXCR7 shRNA was intratracheally administered to sensitized mice 2 weeks before challenge.

**Figure 5. Reduced airway hyperresponsiveness following CXCR7 knockdown.** Airway resistance was measured 48 h after the final challenge by invasive body plethysmography. Data are expressed as the mean  $\pm$  SEM of pulmonary resistance ( $R_L$ ) (n = 3). \*\*\* p < 0.001. Statistical significance was determined by one-way ANOVA. **NC:** negative control, mice sensitized and challenged with PBS. **PC:** positive control, mice sensitized and challenged with OVA. **Lenti-Control:** lentivirus carrying control vector was intratracheally administered to sensitized mice 2 weeks before challenge. **Lenti-CXCR7 shRNA:** lentivirus carrying CXCR7 shRNA was intratracheally administered to sensitized mice 2 weeks before challenge.

**Figure 6. CXCR7 participates in CXCL12-mediated signaling in lung epithelial cells.** (A) Protein extract was isolated from MLE-12 cells, their derived control (bearing control vector, control), and CXCR7 knockdown cell line (bearing CXCR7 shRNA expression construct, CXCR7 shRNA) following stimulation with 100 ng/mL CXCL12 for the indicated time. Western blotting was performed using the indicated antibodies. Quantitative data are shown in supplemental data Figure 2S. (B) (C) Indicated cell lines were loaded with 10 nM/mL Fluo3 and stimulated with 250 ng/mL CXCL12. The changes in relative fluorescence intensity due to calcium influx were assessed. Data are

expressed as the mean  $\pm$  SEM of fluorescence change. Statistical significance was determined by one-way ANOVA.

**Figure 7. Production of CCL2 by airway epithelial cells was regulated by engagement of CXCR7.** (A) The Mouse Cytokine Array Panel A purchased from R&D Systems was utilized to analyze the levels of cytokines in the BALF collected from indicated mice. Indicated cytokine is CCL2. (B) Concentrations of CCL2 in cell culture supernatant collected from MLE-12 cell line stimulated with 100 ng/mL CXCL12 with or without 10  $\mu$ M AMD3100 for 15 h were determined by ELISA using a Duo Set ELISA kit (R&D Systems). (C) Concentrations of CCL2 in the cell culture supernatant collected from MLE-12 cells, their derived control (bearing control vector, p), and CXCR7 overexpression cell line (bearing CXCR7 cDNA expression construct) stimulated with 100 ng/mL CXCL12 for 15 h were analyzed by ELISA. (D) Concentrations of CCL2 in the cell culture supernatant collected from MLE-12, their derived control (bearing control vector), and  $\beta$ -arrestin 2 knockdown cell line (bearing  $\beta$ -arrestin 2 shRNA expression construct) following stimulation with 100 ng/mL CXCL12 for 15 h were analyzed by ELISA. Data were expressed as the mean  $\pm$  SEM of protein concentration. \*\*\*  $p < 0.001$ . Statistical significance was determined by one-way ANOVA.

## Supporting information

**Figure S1 Expression level of CXCR7 in the lung of CXCR7 knockdown mice.** (A) The expression level of CXCR7 in the lung was analyzed by western blot analysis. The protein extracts were prepared from the lungs of OVA challenged BALB/c mice. Band intensity was quantified and analyzed by Image J software after normalization of protein expression to  $\beta$ -actin. **NC:** negative control, mice sensitized and challenged with PBS. **PC:** positive control, mice sensitized and challenged with OVA. **Lenti-Control:** lentivirus carrying control vector was intratracheally administered to sensitized mice 2 weeks before challenge. **Lenti-CXCR7 shRNA:** lentivirus carrying CXCR7 shRNA was intratracheally administered to sensitized mice 2 weeks before challenge.

**Figure S2 Cytokine production was reduced in CXCR7 knockdown mice.** Cytokine production by mediastinal LN cells isolated from mice following re-stimulation with antigen presenting cells and OVA were analyzed by ELISA. In each experiment, mLN cells in the same group were pooled for culturing. Data shown are from two additional independent experiments in addition to Figure 4B. **NC:** negative control, mice sensitized and challenged with PBS. **PC:** positive control, mice sensitized and challenged with OVA. **Lenti-Control:** lentivirus carrying control vector was intratracheally administered to sensitized mice 2 weeks before challenge. **Lenti-CXCR7 shRNA:** lentivirus carrying CXCR7 shRNA was intratracheally administered to sensitized mice 2 weeks before challenge.

**Figure S3 Knockdown efficacy of CXCR7 shRNA in MLE-12 cells.** The expression levels of CXCR7 and CXCR4 protein were determined by western blotting. The protein extract was isolated from MLE-12 cells, their derived control (bearing control vector), and CXCR7 knockdown cell line (bearing CXCR7 shRNA expression construct)

**Figure S4 Quantitative analysis of western blotting in Figure 6A.** Band intensity was quantified and analyzed with Image J software. Data are expressed as the mean  $\pm$  SEM of relative phosphorylation from three independent experiments. \*  $p < 0.05$ . Statistical significance was determined by one-way ANOVA.

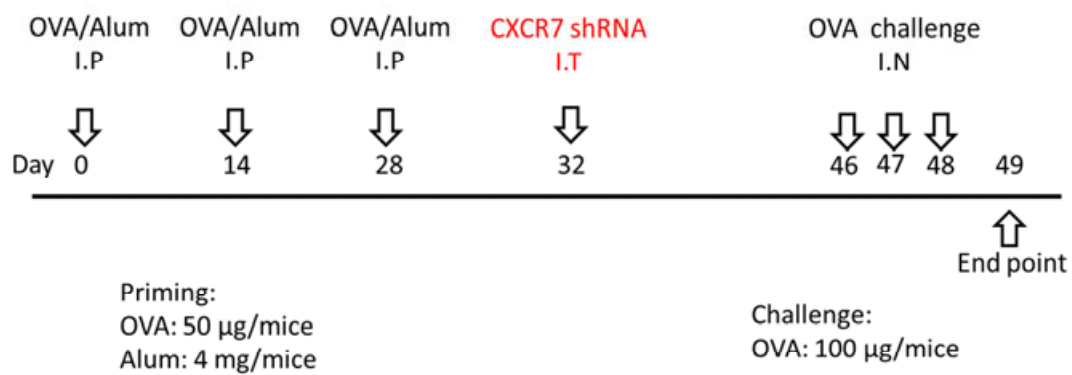


**Figure 1**

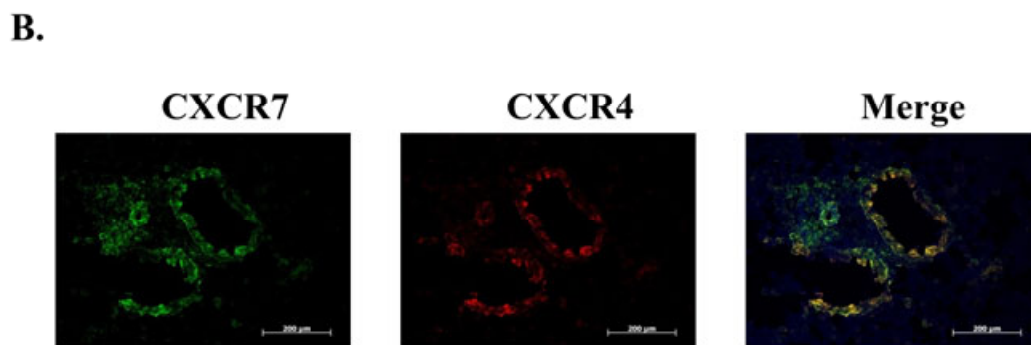
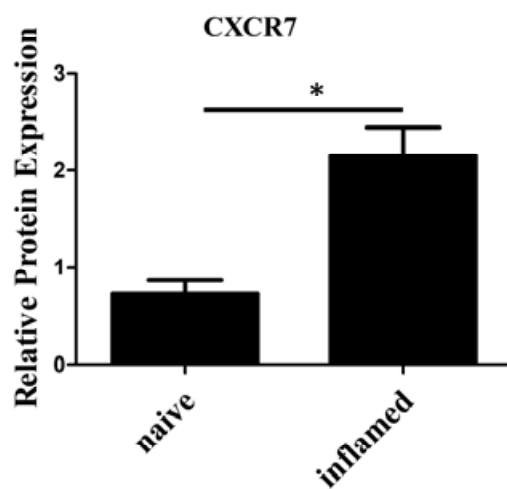
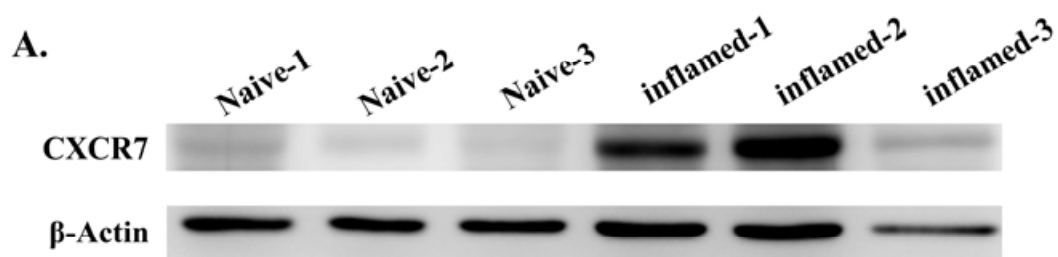


**Figure 2**

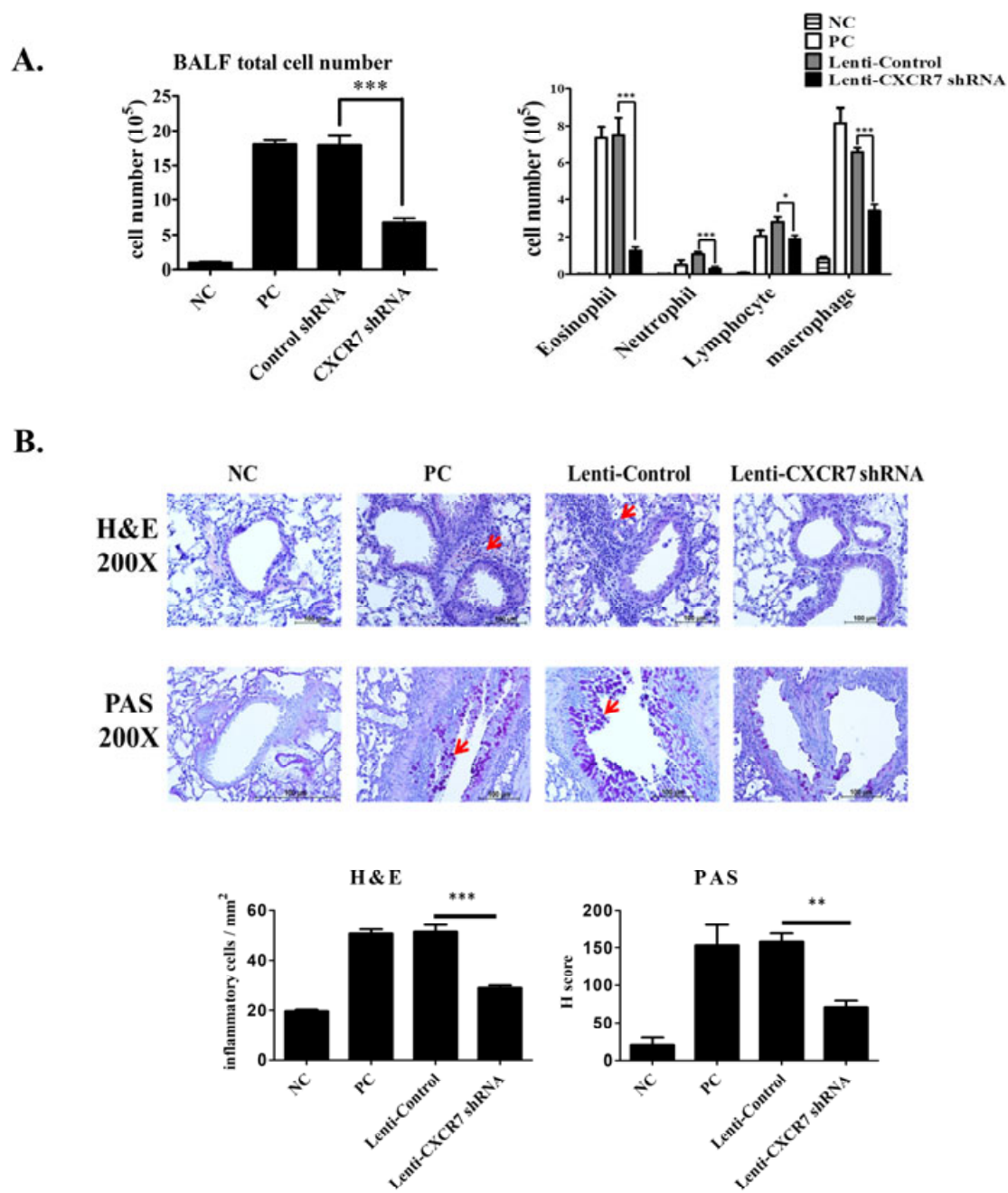
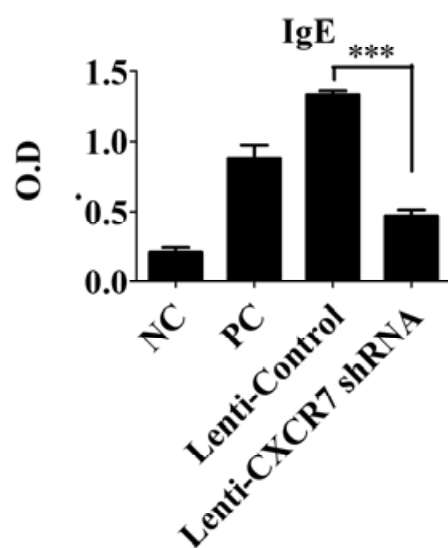


**Figure 3**

A.



B.

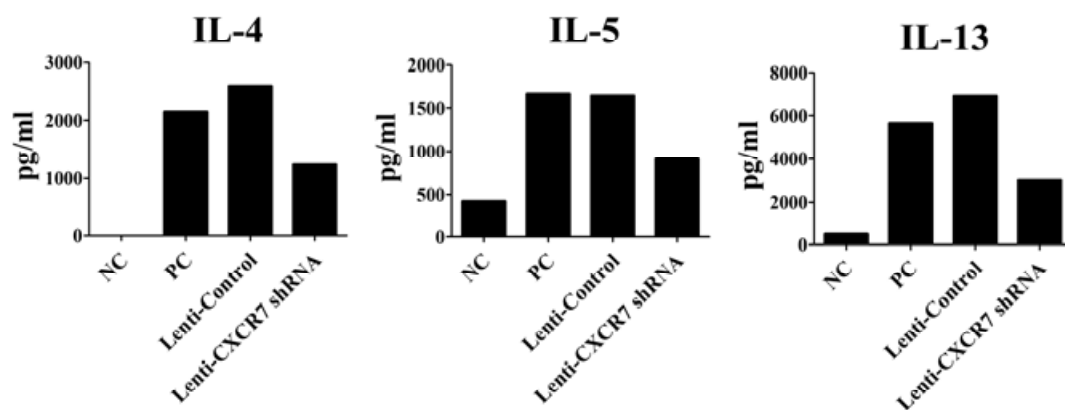


Figure 4

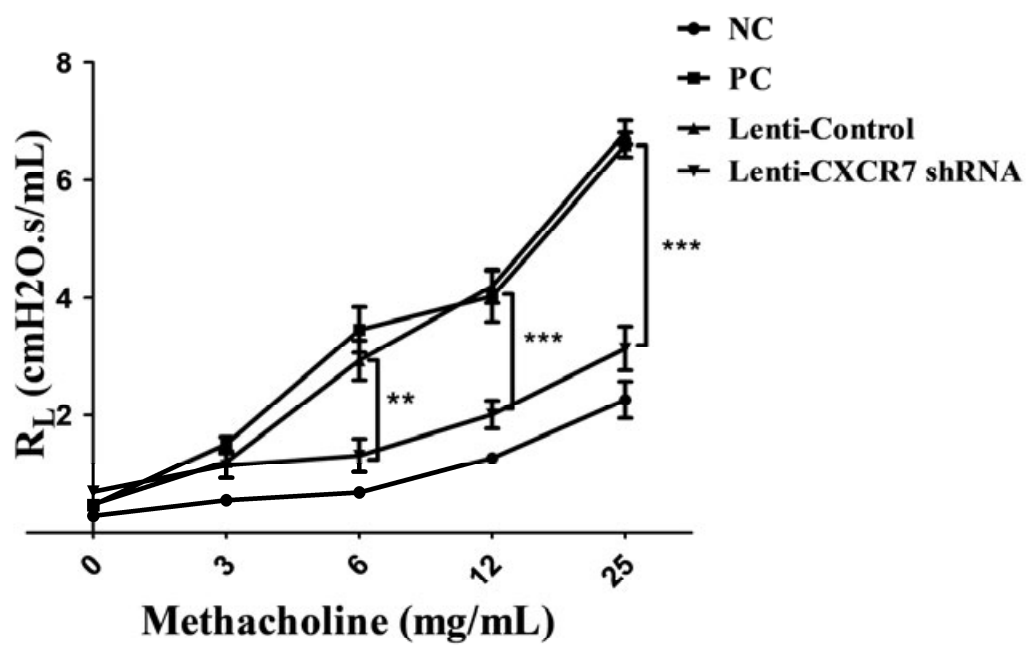
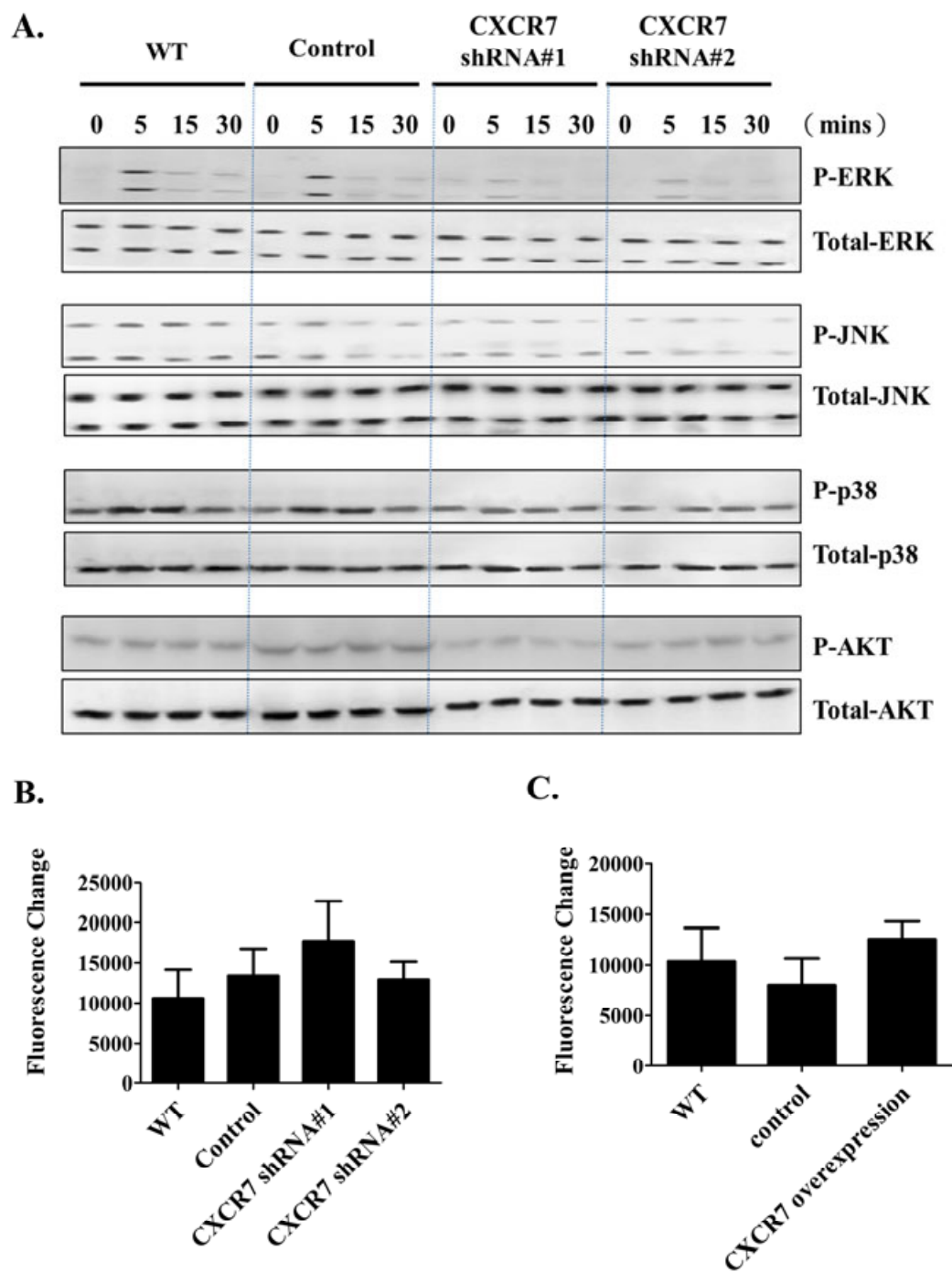
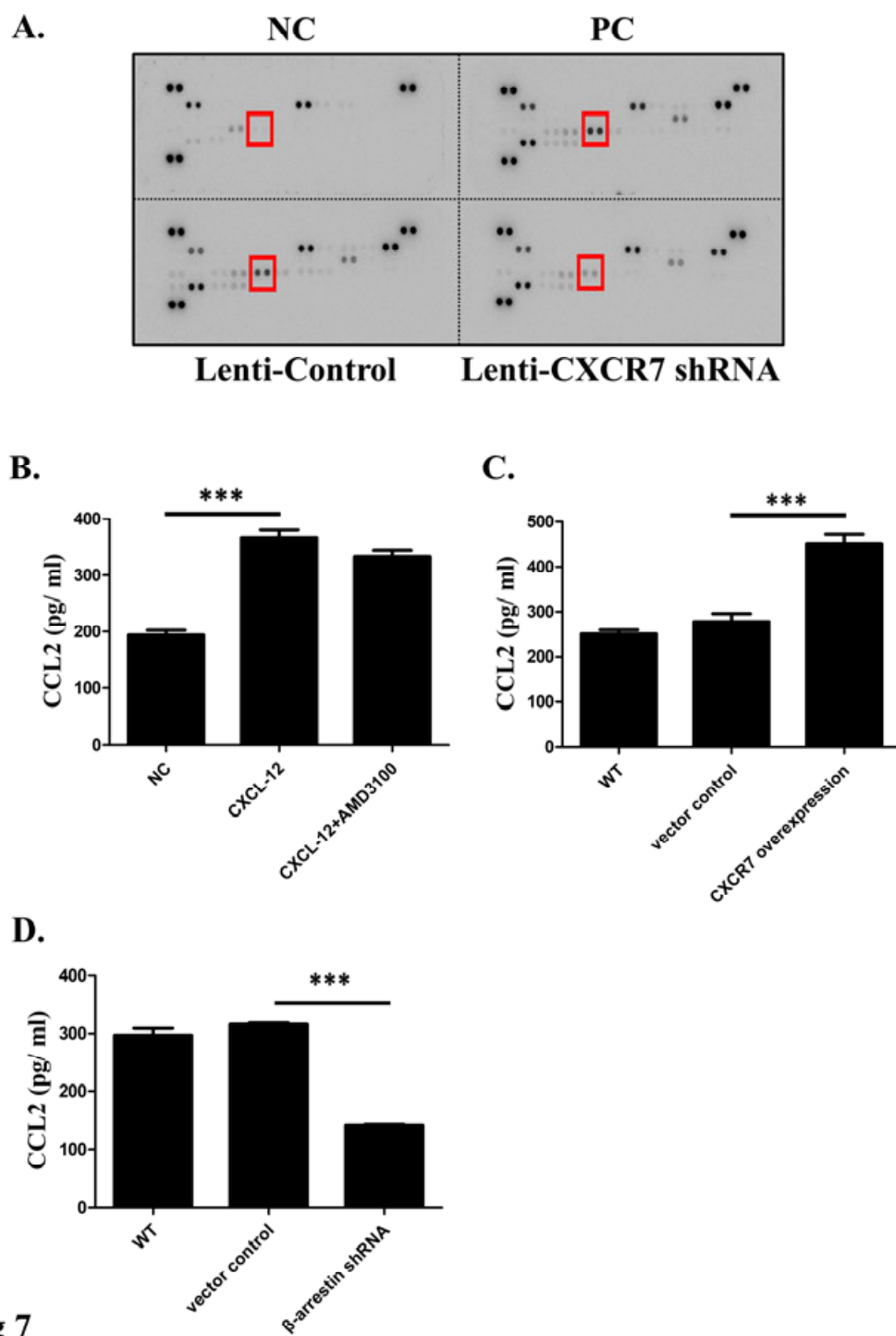


Figure 5

**Fig 6**

**Fig 7**