ORIGINAL RESEARCH

Effects of Differentially Expressed mRNAs Screened Based on GEO Database on Inflammatory Infiltration of Nasal Mucosa in Mice with Allergic Rhinitis

Te Li, MM, Yangfan Wang, MM; Yan Zhao, MM; Rongrong Liu, MD

ABSTRACT

Objective • To identify messenger RNAs (mRNAs) with differential expression in allergic rhinitis (AR) based on an online database, Gene Expression Omnibus (GEO), to provide a new research direction for future diagnosis and treatment of AR.

Methods • The GSE44037 dataset from the CEO database was selected to obtain differentially expressed mRNAs (DEmRNAs) in AR. The keywords involved in these DEmRNAs were enriched and analyzed, and ECM1 and CCL2 were selected for subsequent analysis. In addition, BALB/c mice were purchased and randomized to control (normal feeding), model (AR modeling), si-CCL2 (AR modeling + CCL2 suppression by lentivirus vector), nc-CCL2 (AR modeling + CCL2 empty vector), si-ECM1 (AR modeling + ECM1 suppression by lentivirus vector), and nc-ECM1 (AR modeling + ECM1 empty vector) groups. The frequencies of sneezing and nasal rubbing were recorded in each group. Besides, levels of CCL2, ECM1, interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)-α, and high sensitivity C-reactive protein (hs-CRP) were quantified, and the inflammatory infiltration of nasal mucosa (NM) was observed.

Results • Twenty-six DEmRNAs were acquired from the GSE44037 dataset, among which only CCL2 and ECM1 were found to be associated with keywords such as "immune response" and "inflammatory response" through enrichment analysis. In animal experiments, CCL2 presented lower mRNA expression in model mice than in control mice, while ECM1 showed higher mRNA expression ($P < .05$). The frequencies of sneezing and nose rubbing and the levels of inflammatory factors were significantly increased in si-CCL2 mice compared with model mice, while were significantly decreased in si-ECM1 mice ($P < .05$). The NM inflammatory infiltration was serious in the si-CCL2 group and significantly improved in the si-ECM1 group.

Conclusions • Low expression of CCL2 and high expression of ECM1 in AR are strongly linked to the pathological progression of AR, and these two genes are expected to be new research directions for AR diagnosis and treatment. (*Altern Ther Health Med.* 2023;29(8):608-612).

Te Li, MM; Yangfan Wang, MM; Yan Zhao, MM; Rongrong Liu, MD; Department of Otolaryngology, North-Kuanren General Hospital, Chongqing, China.

Corresponding author: Rongrong Liu, MD
E-mail: lrr00231@163.com

INTRODUCTION

Allergic rhinitis (AR), a non-infectious inflammatory condition of the nasal mucosa (NM) that can be induced by air, dust, food, animal hair and other external factors,[1] has shown a growing incidence in recent years with more than 500 million cases worldwide in 2020.[2] Clinically, AR is mainly manifested as paroxysmal sneezing, watery nasal discharge, itchy nose and nasal congestion, which is also one of the major causes of asthma.[3] Without a clinical cure at present, AR can only be relieved by standardized treatment.[4] As a result, most AR patients without definite allergens experience recurrent attacks and may even develop into severe respiratory dysfunction and failure once they are not treated in time.[5] Although most pathological injuries of AR are not serious, a few patients still died of associated serious respiratory complications. Pathologic studies of AR have confirmed that AR is mainly caused by an imbalance between Th1 and Th2, mediated by immunoglobulin E (IgE) and non-IgE, and belongs to a metaplastic disease characterized by lymphocyte and eosinophil infiltration.[6] Thus, inflammatory infiltration is the most important pathological process in AR, and how to reverse this process is the key to finding new treatment options for AR in the future.

With the medical field's growing attention to human genetic material, exploring diseases' occurrence and development mechanisms through the perspective of genetics has gradually become a research hotspot in modern medicine.[7] AR has also been found to be a chronic

inflammatory change co-acted by genes and environment. For example, NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammatory vesicles accelerate AR progression by activating pyroptosis in macrophages and releasing inflammatory mediators into local tissues.[8] In recent years, many messenger RNAs (mRNAs) have been found to be closely related to AR progression and are involved in inflammatory exacerbation of AR, which provides a novel direction for clinical diagnosis and treatment of AR,[9,10] Therefore, in order to provide more detailed reference and guidance for clinical practice, this study analyzed the differentially expressed mRNAs (DEmRNAs) in AR through the Gene Expression Omnibus (GEO) online database.

MATERIALS AND METHODS

Dataset selection

Public datasets on AR were searched from the GEO database, and GSE44037, a dataset uploaded and made public by Wagener AH in 2013, was selected for analysis, with the analysis platform being GPL13158 [(HT_HG-U133_Plus_PM) Affymetrix HT HG-U133+ PM Array Plate]. A total of 34 sets of specimens were obtained, of which 6 sets of nasal epithelial cells from healthy controls and 5 sets from AR patients were selected as the main analysis targets.

Bioinformatics analysis

GEO2R analysis of GSE44037 was carried out, and DEmRNAs were screened with Log(FC) >1 or < -1 and P<0.05 as the boundary, after which the volcano plot and heat map of these DEmRNAs were drawn. Furthermore, the association network between these DEmRNAs was analyzed by protein-protein interactions (PPIs). Furthermore, the obtained DEmRNAs were clustered and analyzed (based on their overall molecular function) through the pre-built gene annotation databases (e.g., GO and KEGG), and the keywords involving most of the genes were obtained.

Animal data

Ordered from Nanjing Immunophae Technology Co., Ltd. (SYXK [SU] 2022-0052), 30 SPF BALB/c mice (4-6 weeks old, weight 10-12g) were fed and watered freely and kept under a 12:12h light-dark cycle with the temperature maintained at (20 ± 2)°C.

AR model building

Twenty-five mice were randomly selected for AR modeling [10]: 200 μL of normal saline containing 25 μg of ovalbumin (OVA, A5503, Merck) and 2 mg of aluminum hydroxide (1017502, Merck) was injected intraperitoneally into mice every 7 days. Seven days after 3 injections, the mice were given nasal irrigation with 20 μL normal saline containing 3% OVA daily for 2 consecutive weeks. Successful modeling was indicated by clear signs of sneezing, nose rubbing and runny nose, etc. after the treatment described above. The remaining 5 mice were treated as a control group without AR modeling by giving equal amounts of saline intraperitoneal injection and nasal irrigation.

Table 1. Primer sequences

Mouse primers	Forward 3'-5'	Reverse 3'-5'
CCL2	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT
ECM1	TAGTCCTGCCCGTGATGAGT	CCCTTCCACTTCCACAGAGC
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA

Intervention treatment

Jinsili Biotechnology Co., Ltd. was commissioned to construct and design a CCL2-inhibiting lentivirus vector (si-CCL2) and its corresponding empty vector (nc-CCL2), as well as an ECM1-inhibiting lentivirus vector (si-ECM1) and the corresponding empty vector (nc-ECM1). At the last week of modeling, AR model mice were assigned to five groups randomly: one as the model group as the above treatment, and the other four as si-CCL2, nc-CCL2, si-ECM1, and nc-ECM1 group, respectively, for intranasal injection of the corresponding lentivirus vector (1×10^7 IFUs, 2 μL) before nasal administration of OVA for 7 consecutive days.

Behavior observation

After the completion of modeling, the sneezing and nose-rubbing behaviors of mice in each group within 10 minutes were recorded, and the sneezing and nose-rubbing frequencies were calculated.

qRT-PCR

The mice were killed by cervical dislocation under anesthesia. NM tissues were isolated, and total RNA was extracted by TRIzol kit (15596026, Thermo Fisher), 5 μg of which was used for subsequent reverse transcription. Suzhou GENEWIZ Biotech Co. Ltd. was entrusted to design and construct the primer sequences presented in Tab 1. cDNA synthesis system: 2 μg total RNA, 1 μL OligodT, add water to 11 μL, 0.5 μL enzyme, 0.5 μL RNA enzyme inhibitor, 1-2 μL dNTP buffer DEPC water (18080085, Thermo Fisher). The PCR reaction conditions were 95°C, 95°C, and 60°C for 5min, 10s, and 30s, respectively. In this experiment, $2^{-\Delta\Delta CT}$ was utilized to calculate the expression of CCL2 and ECM1 normalized against GAPDH.

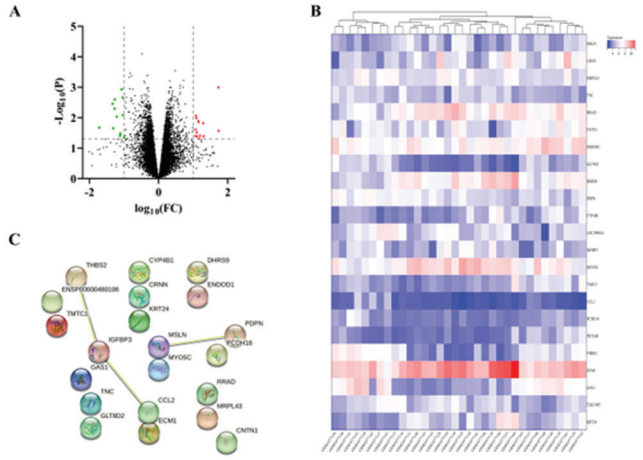
Enzyme linked immunosorbent assay (ELISA)

The levels of interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)-α, and high sensitivity C-reactive protein (hs-CRP) of the serum obtained via centrifugation of mouse carotid blood were detected by ELISA. The kits used were supplied by Beijing Solarbio Science & Technology (SEKM-0013/0016/0047/0024), and the operation process was strictly per the kit manuals.

Statistical analysis

This study used SPSS21.0 for statistical analysis. The results were averaged after three repeated measurements of each experiment, with the results represented by ($\bar{x} \pm s$). The variance analysis and Bonferroni test were used for comparison among multiple groups of data, with the presence of significance indicated by $P < .05$.

Figure 1. Differential gene expression. **A.** The volcanic plot of differentially expressed mRNAs. **B**. The heat map of differentially expressed mRNAs. **C.** PPI networks.



RESULTS

Differential gene expression

Twenty-six DEmRNAs were identified in the GSE44037 dataset (Figure 1-A, B), including significantly underexpressed mRNAs such as LOC389834 and IGFBP3 and obviously highly expressed mRNAs like PICSAR and CRNN. The PPI network is presented in Figure 1-C, which reveals a certain correlation between THBS2, GAS1, and CCL-2 and MSLN and PDPN.

Enrichment analysis

According to the KEGG analysis of the 26 DEmRNAs, there were 4 keywords involving multiple genes, among which "Malaria", "ECM-receptor interaction" and "Focal adhesion" were the keywords with statistically significant differences ($P < .05$). In the GO analysis, the keywords concerning the 26 DEmRNAs included "extracellular space", "biological adhesion" and "cell adhesion". Further, we focused on mRNAs related to "immune response" and "inflammatory response" in the above-mentioned DEmRNAs, and selected CCL2 and ECM1 for follow-up research as only these two genes were identified to be related to the above keywords (Figure 2).

Modeling results

One mouse each in si-CCL2 and nc-ECM1 groups died. Another mouse died after nasal administration, presumably due to suffocation caused by too fast injection during nasal administration. The other one died in the cage with obvious bite scars, which was presumed to be caused by the attack of mice housed in the same cage.

Detection of CCL2 and ECM1 expression

After detection, CCL2 mRNA expression in model mice was found to be (1.40±0.13), lower than that in control mice ($P < .05$), while ECM1 mRNA was (1.88 ± 0.17), which was higher compared with control mice ($P < .05$). In addition, mRNA levels of CCL2 and ECM1 in si-CCL2 and si-ECM1 groups were lower than those in the corresponding nc-CCL2 and nc-ECM1 groups ($P < .05$), confirming the successful intervention of lentiviral vector that inhibited gene expression (Figure 3).

Figure 2. Enrichment analysis. **A.** KEGG analysis. **B.** GO analysis.

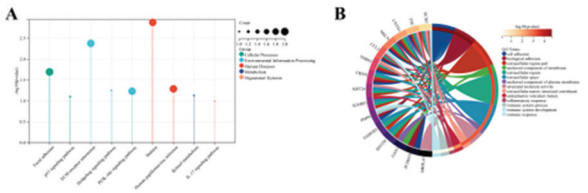
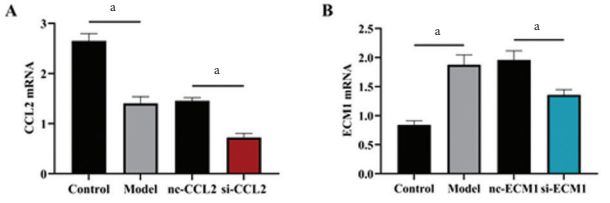
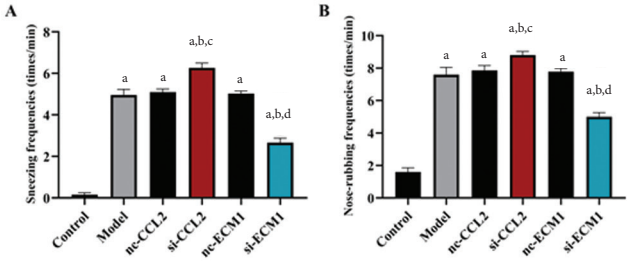


Figure 3. Detection of CCL2 and ECM1 expression. **A.** Comparison of CCL2 mRNA. **B**. Comparison of ECM1 mRNA.



[a]statistically significant difference between the two groups ($P < .05$).

Figure 4. Observation of mouse behavior. **A.** Comparison of sneezing frequencies. **B.** Comparison of nose-rubbing frequencies.



[a]statistically significant difference between and control group ($P < .05$)
[b]statistically significant difference between and Model group ($P < .05$)
[c]statistically significant difference between and nc-CCL2 group ($P < .05$)
[d]statistically significant difference between and nc-ECM1 group ($P < .05$)

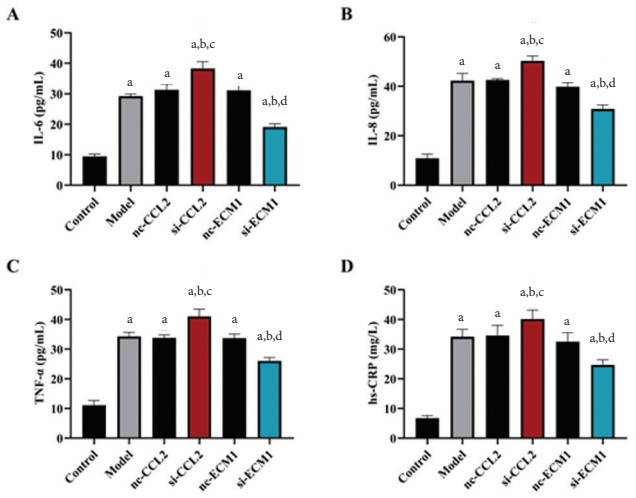
Observation of mouse behavior

Model mice showed higher sneezing and nose-rubbing frequencies than control mice ($P < .05$). Among the other four mouse groups, the si-CCL2 group had the highest sneezing and nose-rubbing frequencies, higher than the model group ($P < .05$), while the si-ECM1 group had the lowest sneezing and nose-rubbing frequencies, still higher than the control group ($P < .05$). The sneezing and nose-rubbing frequencies in nc-CCL2 and nc-ECM1 groups were not significantly different from those in the model group ($P > .05$) (Figure 4).

Detection results of inflammatory factors (IFs)

As shown in Figure 5, model mice had higher IL-6, IL-8, TNF-α, and hs-CRP levels than control mice ($P < .05$); the

Figure 5. Detection results of IFs. **A**. Comparison of IL-6. **B**. Comparison of IL-8. **C.** Comparison of TNF-α. **D**. Comparison of hs-CRP.



[a]statistically significant difference between and control group ($P < .05$)
[b]statistically significant difference between and Model group ($P < .05$)
[c]statistically significant difference between and nc-CCL2 group ($P < .05$)
[d]statistically significant difference between and nc-ECM1 group ($P < .05$)

levels of these IFs in nc-CCL2 and nc-ECM1 groups were also no different from those in the model group ($P > .05$); while IL-6, IL-8, TNF-α and hs-CRP levels in the si-ECM1 group were lower than those in the model group ($P < .05$), but higher than those in the control group ($P < .05$); and higher levels of these IFs were observed in the si-CCL2 group compared with the model group ($P < .05$).

DISCUSSION

As a non-infectious disease involving various immune cell activation and chronic inflammatory lesions, AR is released by IgE-mediated mediators after allergen exposure in atopic individuals, with various immunocompetent cells and cytokines involved.[11] How to regulate the release of IgE-mediated downstream substances may be the key to curing AR in the future. Regulating human proteins and cell life behaviors through mRNA pathways is a hot topic in the medical field, and a thorough understanding of AR-related mRNAs is the basis for confirming molecular targeted therapies. This study identified DEmRNAs in AR through an online database with important clinical implications.

This study found 26 DEmRNAs in the GSE44037 dataset. Through enrichment analysis, only CCL2 and ECM1 were confirmed to be associated with immune response and inflammatory response, the key to the pathogenesis and pathological progression of AR.[12] Therefore, we focus on CCL2 and ECM1 and carried out further analysis.

First, we established an AR mouse model through OVA induction, a classic AR modeling method in clinic with its effectiveness repeatedly verified.[13,14] The high modeling efficiency of this experiment was confirmed, with only one mouse dying because of improper manipulation. Subsequently, the observation of mouse behavior showed significantly higher sneezing and nasal-rubbing frequencies as well as significantly aggravated levels of IFs and pathological damage of NM in model mice compared with controls. In a previous study on AR mouse models, researchers also confirmed inflammation of the nasal mucosal pathology injury and elevated levels of inflammatory factors in AR mice, which is consistent with our results[15] and confirms the success of AR model establishment. We also observed a decrease in CCL2 and an increase in ECMI in AR model mice. This is in line with the bioinformatics analysis results, suggesting the involvement of CCL2 and ECM1 in the occurrence and development of AR. CCL2, the most well-known chemokine with chemotactic activity against monocytes and basophils, is the key to regulating monocyte/macrophage migration and infiltration.[16] At present, studies on CCL2 mainly focus on malignant neoplastic diseases.[17,18] Moreover, most autoimmune diseases are characterized by the infiltration of lymphocytes into target tissues, leading to inflammation and tissue damage, a process in which CCL2 acts as a signaling bridge in a complex network of immune cells.[19] It has also been demonstrated that CCL2 in psoriasis can be co-amplified by TNF-α and interferon-γ (IFN-γ),[20] showing its important potential impact on immune diseases such as AR. Sobkowiak P et al. reported under-expressed CCL2 in 21 children with AR and 39 children with allergic asthma,[21] similar to our findings. ECM1, as a secreted glycoprotein, is one of the components of the normal extracellular matrix, which plays a vital regulatory role in T cell differentiation and function.[22] In recent years, ECM1 has also been widely concerned in the study of various inflammatory diseases as it enhances the M1 polarization of macrophages.[23] Although the expression of ECM1 in AR has not been confirmed yet, ECM1 is uniformly up-regulated in acute lung injury and ulcerative colitis,[24,25] which is also of great reference value to our research results and can preliminarily support its potential relationship with AR.

To further confirm the role played by CCL2 and ECM1 in AR, we interfered with their expression in AR mice by injecting corresponding lentivirus vectors. It was subsequently found that after silencing CCL2, the sneezing and nasal-rubbing frequencies and the levels of IFs in AR mice were further increased, and the NM damage was exacerbated. However, after ECM1 was silenced, there was a marked improvement in the pathological manifestations in the mice. It is suggested that down-regulating CCL2 can promote the pathological damage of AR, while silencing ECM1 can alleviate it. The above experiments verify the expression patterns of CCL2 and ECM1 in AR and lay a reliable foundation for these two as therapeutic targets for AR in the future. Similarly, Zhang Y et al. also considered CCL2 as a new direction for future AR treatment,[26] which can verify our point of view.

But due to limited conditions, we have not been able to analyze the influences of CCL2 and ECM1 on the biological behavior of NM epithelial cells in AR mice. Besides, the correlation of the two genes with AR needs further validation by

constructing vectors that increase gene expression. And given that many potential AR-related mRNAs are still yet to be discovered, we will conduct more in-depth research to provide a more comprehensive reference for AR diagnosis and treatment.

CONCLUSION

Through online database analysis, we found 26 DEmRNAs in AR, of which CCL2 was under-expressed and ECM1 was over-expressed, showing a close relationship with immune and inflammatory responses through enrichment analysis. Through establishing an AR mouse model, silencing CCL2 was confirmed to be able to promote the inflammatory infiltration of NM in AR mice, while silencing ECMI alleviated the pathological progression of AR. Thus, molecular targeting of CCL2 or ECM1 may be a new direction for future AR therapies.

REFERENCES

1. Hoyte FCL, Nelson HS. Recent advances in allergic rhinitis. *F1000 Res*. 2018;7:7. doi:10.12688/f1000research.15367.1
2. Bousquet J, Anto JM, Bachert C, et al. Allergic rhinitis. *Nat Rev Dis Primers*. 2020;6(1):95. doi:10.1038/s41572-020-00227-0
3. Siddiqui ZA, Walker A, Pirwani MM, Tahiri M, Syed I. Allergic rhinitis: diagnosis and management. *Br J Hosp Med (Lond)*. 2022;83(2):1-9. doi:10.12968/hmed.2021.0570
4. Okubo K, Kurono Y, Ichimura K, et al; Japanese Society of Allergology. Japanese guidelines for allergic rhinitis 2020. *Allergol Int*. 2020;69(3):331-345. doi:10.1016/j.alit.2020.04.001
5. Schuler Iv CF, Montejo JM. Allergic Rhinitis in Children and Adolescents. *Immunol Allergy Clin North Am*. 2021;41(4):613-625. doi:10.1016/j.iac.2021.07.010
6. Zhang Y, Lan F, Zhang L. Advances and highlights in allergic rhinitis. *Allergy*. 2021;76(11):3383-3389. doi:10.1111/all.15044
7. Yamamoto M, Curiel DT. Cancer gene therapy. *Technol Cancer Res Treat*. 2005;4(4):315-330. doi:10.1177/153303460500400402
8. Choi BY, Han M, Kwak JW, Kim TH. Genetics and Epigenetics in Allergic Rhinitis. *Genes (Basel)*. 2021;12(12):2004. doi:10.3390/genes12122004
9. Wei X, Zhang B, Liang X, et al. Higenamine alleviates allergic rhinitis by activating AKT1 and suppressing the EGFR/JAK2/c-JUN signaling. *Phytomedicine*. 2021;86:153565. doi:10.1016/j.phymed.2021.153565
10. Jin P, Zhang H, Zhu X, et al. Bioinformatics analysis of mRNA profiles and identification of microRNA-mRNA network in $CD4^+$ T cells in seasonal allergic rhinitis. *J Int Med Res*. 2022;50(8):3000605221113918. doi:10.1177/03000605221113918
11. Agnihotri NT, McGrath KG. Allergic and nonallergic rhinitis. *Allergy Asthma Proc*. 2019;40(6):376-379. doi:10.2500/aap.2019.40.4251
12. Meng Y, Wang C, Zhang L. Advances and novel developments in allergic rhinitis. *Allergy*. 2020;75(12):3069-3076. doi:10.1111/all.14586
13. Madanipour MR, Fatehi-Zardalou M, Rahimi N, et al. The anti-inflammatory effect of dapsone on ovalbumin-induced allergic rhinitis in balb/c mice. *Life Sci*. 2022;297:120449. doi:10.1016/j.lfs.2022.120449
14. You X, Sun X, Kong J, Tian J, Shi Y, Li X. D-Pinitol Attenuated Ovalbumin-induced Allergic Rhinitis in Experimental Mice via Balancing Th1/Th2 Response. *Iran J Allergy Asthma Immunol*. 2021;20(6):672-683. doi:10.18502/ijaai.v20i6.8017
15. Shi Z, Jiang W, Chen X, et al. Chlorogenic acid ameliorated allergic rhinitis-related symptoms in mice by regulating Th17 cells. *Biosci Rep*. 2020;40(11):BSR20201643. doi:10.1042/BSR20201643
16. Xu M, Wang Y, Xia R, Wei Y, Wei X. Role of the CCL2-CCR2 signalling axis in cancer: mechanisms and therapeutic targeting. *Cell Prolif*. 2021;54(10):e13115. doi:10.1111/cpr.13115
17. Hao Q, Vadgama JV, Wang P. CCL2/CCR2 signaling in cancer pathogenesis. *Cell Commun Signal*. 2020;18(1):82. doi:10.1186/s12964-020-00589-8
18. Yang H, Zhang Q, Xu M, et al. CCL2-CCR2 axis recruits tumor associated macrophages to induce immune evasion through PD-1 signaling in esophageal carcinogenesis. *Mol Cancer*. 2020;19(1):41. doi:10.1186/s12943-020-01165-x
19. Miyamoto T, Murakami R, Hamanishi J, et al. B7-H3 Suppresses Antitumor Immunity via the CCL2-CCR2-M2 Macrophage Axis and Contributes to Ovarian Cancer Progression. *Cancer Immunol Res*. 2022;10(1):56-69. doi:10.1158/2326-6066.CIR-21-0407
20. Zhang F, Mears JR, Shakib L, et al; Accelerating Medicines Partnership Rheumatoid Arthritis and Systemic Lupus Erythematosus (AMP RA/SLE) Consortium. IFN-γ and TNF-α drive a CXCL10+ CCL2+ macrophage phenotype expanded in severe COVID-19 lungs and inflammatory diseases with tissue inflammation. *Genome Med*. 2021;13(1):64. doi:10.1186/s13073-021-00881-3
21. Sobkowiak P, Narożna B, Wojsyk-Banaszak I, Bręborowicz A, Szczepankiewicz A. Expression of proteins associated with airway fibrosis differs between children with allergic asthma and allergic rhinitis. *Int J Immunopathol Pharmacol*. 2021;35:2058738421990493. doi:10.1177/2058738421990493
22. Fan W, Liu T, Chen W, et al. ECM1 Prevents Activation of Transforming Growth Factor β, Hepatic Stellate Cells, and Fibrogenesis in Mice. *Gastroenterology*. 2019;157(5):1352-1367.e13. doi:10.1053/j.gastro.2019.07.036
23. Zhang Y, Li X, Luo Z, et al. ECM1 is an essential factor for the determination of M1 macrophage polarization in IBD in response to LPS stimulation. *Proc Natl Acad Sci USA*. 2020;117(6):3083-3092. doi:10.1073/pnas.1912774117
24. Yang L, Brooks CR, Xiao S, et al. KIM-1-mediated phagocytosis reduces acute injury to the kidney. *J Clin Invest*. 2015;125(4):1620-1636. doi:10.1172/JCI75417
25. Thompson AI, Lees CW. Genetics of ulcerative colitis. *Inflamm Bowel Dis*. 2011;17(3):831-848. doi:10.1002/ibd.21375
26. Zhang Y, Zhu K, Xia C, et al. Multiple-Cytokine Profiling: A Novel Method for Early Prediction of the Efficacy of Sublingual Immunotherapy in Allergic Rhinitis Patients. *J Inflamm Res*. 2022;15:603-612. doi:10.2147/JIR.S350003