

# CCL20/CCR6 Mediated Macrophage Activation and Polarization Can Promote Adenoid Epithelial Inflammation in Adenoid Hypertrophy

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**Background:** Adenoid hypertrophy (AH) is a chronic or acute obstruction-related ailment of the upper respiratory tract that arises as an inflammatory response to exposure of bacteria, viruses or allergies. Activation and polarization of macrophages are key processes in inflammation-related disorders like AH and CCL20/CCR6 axis is a critical therapeutic target.

**Purpose:** To determine that CCL20/CCR6 mediated macrophage activation and polarization can promote adenoid epithelial inflammation in AH.

**Methods:** To support this claim, CCL20 and CCR6 expressions were studied in clinical AH samples. In addition, the expressions of cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-17, IL-10 and TGF- $\beta$  were analysed. In vitro, human adenoid epithelial cells were co-cultured with polarized THP-1 and T lymphocyte H9 cells to study the expressions of several inflammatory markers.

**Results:** The expressions of M1 macrophage markers CD86 and IL-17 were significantly increased, whereas the expressions of M2 macrophage markers CD206 and FOXP3 were significantly decreased. The THP-1 cells were successfully polarized to M0, M1 and M2 macrophages. The survival of macrophages improved after 24 hr of induction and enhanced TGF- $\beta$  expression was observed. The expressions of the inflammatory cytokines IL-6, TNF- $\alpha$ , IL-1 $\beta$  and CCL20 increased significantly.

**Conclusion:** Collectively, these results suggest that the CCL20/CCR6 mediated macrophage activation and polarization into M1-type macrophages can promote adenoid epithelial inflammation in AH. Further studies are warranted to determine the roles of inflammatory markers in the pathophysiology of AH and identifying potential targets.

**Keywords:** adenoid hypertrophy, CCL20/CCR6 axis, inflammation, macrophages, markers

## Introduction

Adenoids are condensed forms of lymphoepithelial tissues existing behind the nose in the posterosuperior region of nasopharynx, medial to the Eustachian tube orifices and are responsible for enhanced immunological memory recognized amidst children.<sup>1,2</sup> Adenoid hypertrophy (AH) is a chronic or acute obstruction-related ailment that arises as an inflammatory response and is observed as a blockage of flow of air via the upper nasal region.<sup>3</sup> It is commonly witnessed among children of ages less than 16, where the adenoids enlarge reaching its peak at age 6.<sup>4</sup> Several viral and bacterial pathogens have been identified to be infectious signatures for AH.<sup>5</sup> Passive or involuntary exposure to cigarette smoke, occurrences of allergy and gastroesophageal reflux are associated with non-infectious risk factors for AH.<sup>6</sup> Since changes in the immunological barrier can be critical among children who suffer from obstruction of the upper nasal region, sleep apnea and otitis media, diagnosis remains significant for observing the adenoids. Procedures such as posterior rhinoscopy, nasal endoscopy and investigations using radiological examinations such as computed tomography and magnetic resonance imaging are usually preferred for the diagnosis of obstructive AH. Other objective tests like computed

rhinomanometry and acoustic rhinometry are also used to serve this purpose. Adenoidectomy is considered quite a familiar surgical procedure for management of the condition.<sup>7–9</sup>

Monocytes and macrophages form the core of innate immunity and maintaining the homeostasis among these cells can protect us from self- and external antigens. These immune cells can engulf bacteria by phagocytosis via the secretion of mediator components responsible for pro-inflammatory and antimicrobial effects.<sup>10,11</sup> Monocytes can spot danger signals and differentiate into terminal macrophages which can ingest pathogenic microbes and their toxins and yield the processed antigens to other cells of the immune system.<sup>12</sup> In addition, studies conducted on clinical adenoid samples of AH patients indicate that elevated serum enzyme or protein levels that resemble the activation of monocyte/macrophage and myeloid cells like eosinophils have been attained.<sup>13</sup> Interestingly, bacterial pathogens that can cause infections of the upper respiratory tract were found intracellularly in monocytes/macrophages of adenoids from children with AH.<sup>14</sup> Also, macrophages and monocytes that display pro-inflammatory markers like CD14 and CD64 are observed extensively in children with AH.<sup>15,16</sup>

Macrophage inflammatory protein 3 (MIP-3 $\alpha$ ), better known as Chemokine CCL20, is upregulated under the action of inflammatory mediators, thereby regulating the immune function of the body.<sup>17</sup> Moreover, CCR6 is a G protein-coupled receptor expressed in various immune cells and has the ability to specifically interact with its ligand CCL20.<sup>18</sup> CCL20-CCR6 axis is extensively studied in inflammation-related diseases like cancer, diabetes and several other disorders, whereas, less studied with regard to AH.<sup>19–21</sup> The effect of CCL20 on its receptor CCR6 in T lymphocytes can induce cell differentiation into Th17, and the increase of Th17/Treg ratio is recognized as an inducer of AH.<sup>22,23</sup> However, the effect of macrophage activation and polarization mediated by CCL20/CCR6 axis on adenoid epithelial inflammation and lymphocyte differentiation in AH has not been reported so far.

Therefore, in the present report, we will first detect the levels of CCL20, CCR6 and macrophage marker proteins (different activation and polarization phenotypes) and verify the actual expression of these factors in diseased samples. Following this, the expressions of the above factors was regulated by human intervention via assays using cell line models so as to verify the relevant influencing mechanisms and relationships.

## Materials and Methods

### Collection of Clinical Samples

All procedures and protocols were approved by the Ethics Committee of Affiliated Hospital of Nanjing University of Chinese Medicine and Yixing Hospital of Traditional Chinese Medicine. All procedures employed in the study using human participants complied with the Helsinki Declaration and its amendments. Free and informed consent to contribute to this study was acquired from the parents or legal guardian for the 19 children under legal age to consent. Diagnosis was done by electronic nasopharyngoscopy or sella turcica lateral X-ray examination. The study participants or children with hypertrophy underwent bilateral tonsil adenoidectomy for obstructive complications such as snoring, nasal congestion, sleep apnea, open mouth breathing or adenoid visage. The excised adenoids were stored at  $-80^{\circ}\text{C}$  or fixed with 4% polyoxymethylene for further use. The general information of clinical adenoid samples acquired from AH patients is shown in Table 1.

### ELISA

To examine the expressions of CCL20 and CCR6 en route of detecting macrophage polarization among adenoid tissues, ELISA test was performed. Subsequently, 5 samples with the high CCL20 expressions (designated as HE group with enlarged adenoids) and 5 samples with the low expressions of CCL20 (designated as LE group with comparatively lesser enlargement of the adenoids) were selected for further pathological examination. CCL20/CCR6 protein expression profiles in the obtained adenoids were quantified by adopting enzyme-linked immunosorbent assay (ELISA) with human CCL20 and CCR6 kits adhering to the instructions provided by the manufacturer (Jianglaibio, China). The absorbance was read using a Multiscan Ex ELISA microplate reader (Thermo Fisher). The expressions of IL-1 $\beta$ , IL-6, IL-17, IL-10, TNF- $\alpha$  and TGF- $\beta$  were quantified through equivalent kits obtained from Beyotime Inc., China.

**Table I** Relevant Information of the Clinical Samples from AH Patients

Sample	Gender	Age (Years)	Duration of Lesion (in Months)	CCL20 (pg/mL)	CCR6 (pg/mL)
1	Male	5	24	195.7	55.1
2	Female	5	6	236.2	64
3	Male	6	36	245.6	67.6
4	Male	3	6	246.6	68.4
5	Male	6	12	247.7	72.1
6	Male	8	36	237.3	69.2
7	Female	3	6	252.9	73.4
8	Female	8	48	214.4	60.6
9	Female	7	24	259.1	71.5
10	Male	6	24	298.7	80.7
11	Female	5	12	248.7	73.4
12	Male	7	24	245.6	64.8
13	Male	7	36	240.4	67.6
14	Male	9	24	252.9	74.2
15	Female	5	12	269.5	75.5
16	Female	12	72	284.1	81.7
17	Male	11	48	274.7	71.3
18	Male	12	24	283.1	72.3
19	Male	11	48	273.7	79.9

## Hematoxylin and Eosin Staining

The adenoids were initially fixed with 4% polyoxymethylene for 24 hr at 37°C. Later, the tissues were paraffin-embedded, sectioned into slices of 5 µm thickness and stained with the two histological stains obtained from Nanjing Jiancheng Bioengineering Institute, China. The sections were dried out, resin-sealed and randomly selected fields were used for capturing the images by Nikon inverted fluorescent microscope (200X).

## Immunohistochemical (IHC) Staining

The expressions of macrophage markers (CD86 and CD206) and Treg and Th17-associated markers (IL-17A and FOXP3) in the adenoid tissues were evaluated by IHC according to established protocols. Paraffin sections were prepared by heating in an oven for 1 hr. The sections were later directed towards xylene-based deparaffinization followed by ethanol-centred rehydration and heated by means of microwave oven using sodium citrate buffer. The slides were later allowed for cooling at room temperature and immersed in 3% H<sub>2</sub>O<sub>2</sub> for 20 min. The tissue segments were smeared with blocking buffer containing 3% BSA and incubated with 50 µL of primary antibodies overnight at 4°C. After a PBS wash, the sections were incubated with secondary antibodies for 20 min at 37°C. The samples were then colored by diaminobenzidine (DAB), washed and stained with hematoxylin, diluted for 30 secs with HCl, dehydrated, sealed and observed under a microscope.

## Cell Culture

Human adenoid epithelial cells were taken from human adenoids and cultured with RPMI1640 containing 10% FBS containing 1% penicillin plus streptomycin (Gibco; Thermo Fisher Scientific, Inc., USA) under humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. Human THP-1 and T lymphocyte H9 cells (American Type Culture Collection) were cultured under similar conditions as mentioned before. To induce the polarization of THP-1 cells, it was first treated with PMA (10ng/mL, Sigma-Aldrich). The THP-1 cells were later processed with IFN- $\gamma$  (20 ng/mL, Sigma-Aldrich) and lipopolysaccharide (LPS, 100 ng/mL, Sigma-Aldrich) for M1 polarization. For M2 polarization, the cells were treated with IL-4 (20 ng/mL, Sigma-Aldrich) and IL-13 (20 ng/mL, Sigma-Aldrich). The control group did not receive any special processing. The polarized cell culture medium was used to cultivate H9 cells, and the cells were grouped into control, M0, M1 and M2 groups. Accordingly, the polarized cell culture medium was used to cultivate adenoid epithelial cells, and the cells induced by M1 medium were treated with CCR6 inhibitors. The cells were grouped into control, M0, M1, M2 and M1+CCR6 inhibitor groups.

## Cell Counting Kit-8 (CCK-8) Cell Viability Assay

H9 and adenoid epithelial cells ( $8 \times 10^3$ /well) were cultured and after induction with corresponding conditioned medium, 10  $\mu$ L of CCK-8 reagent obtained from Dojindo Laboratories, Japan, was appended and incubated for 2 hr. The absorbance was later measured at 450 nm using a microplate reader (Thermo Scientific, USA).

## Western Blotting

Cultured cells were lysed in cell RIPA lysis buffer, and a bicinchoninic acid (BCA) kit was used to detect the protein concentration (Beyotime Inc., China). Equal amounts of protein (20  $\mu$ g) were sorted using 10% SDS-PAGE and moved on to PVDF membranes (Invitrogen; Thermo Fisher Scientific, Inc., USA). Later, the membranes were incubated with the following primary antibodies at 4°C overnight: anti-CCR6 (1:1000, ab227036, Abcam); anti-NLRP3 (1:1000, 263899, Abcam); anti-cleaved-Caspase-1 (1:1000, 4199, CST); anti-Caspase-1 (1:1000, 98033, CST); anti-GSDMD-N (1:1000, ab215203, Abcam); anti-GAPDH (1:1000, ab9485, Abcam). Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (1:5000, ab150077, Abcam) were then used to treat the membrane for 1 hr at room temperature. Thereafter, the bands of proteins were envisaged using ECL-prime kit and analyzed with ImageJ program.

## Immunofluorescence (IF) Staining

THP-1 cells were treated with PMA, IFN- $\gamma$  + LPS and IL-4 + IL-13, respectively, and affixed with 4% polyoxymethylene (Sigma-Aldrich) at 4°C for 15 min. Subsequently, the cells were treated with 1% Triton X-100 in 0.01 M PBS for 15 min. Antibodies for CD14, CD11b, CD86 and CD206 (Abcam, 1:50, Cambridge, MA) were incubated with the cells at 4°C the full night. After treating with the primary antibodies, Alexa Fluor 546 conjugated goat anti-rabbit IgG (1:200; Invitrogen) was used to visualize the samples using a fluorescence microscope (Olympus, IX71, Japan).

## Statistical Analyses

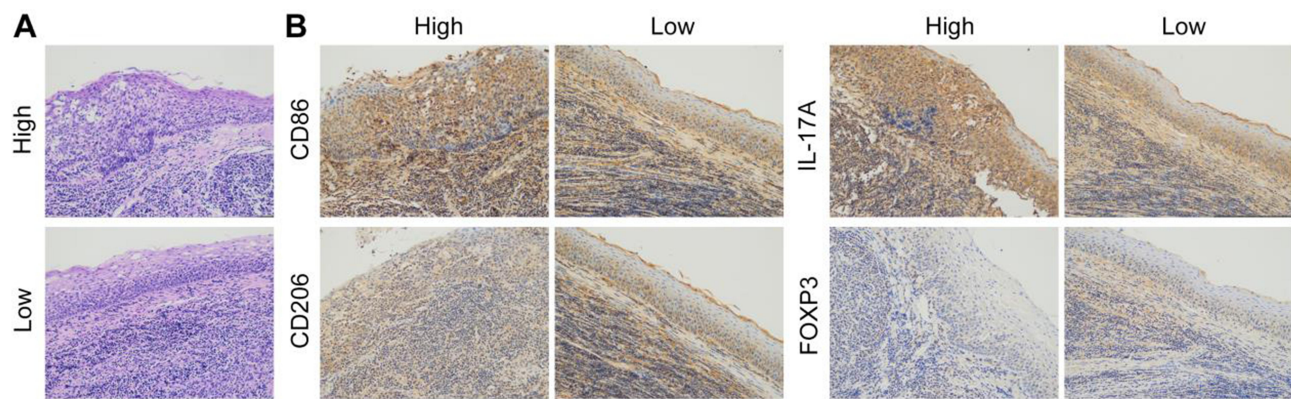
The difference among groups were represented statistically as mean  $\pm$  SD. One-way analysis of variance (ANOVA) by means of Tukey's test was used as a tool to make multiple comparisons. *P* value <0.05 was considered to be statistically significant among the triplicates tested. All experiments were performed in triplicate, and the data are presented as mean  $\pm$  SD.

## Results

### The CCL20/CCR6 Axis Mediated Macrophage Polarization and Lymphocyte Differentiation in AH Samples

To examine the expressions of CCL20 and CCR6 en route of detecting macrophage polarization among adenoid tissues, ELISA test was performed. It was interesting to find that the samples with high CCL20 content had obvious AH as observed via enlarged adenoids, while the AH patients with low CCL20 expressions had comparatively lesser enlargement of the adenoids (Figure 1A). Subsequently, 5 samples with the high CCL20 expressions (HE group) and 5 samples with the low expressions of CCL20 (LE group) were selected for further pathological examination. To accomplish this purpose, the



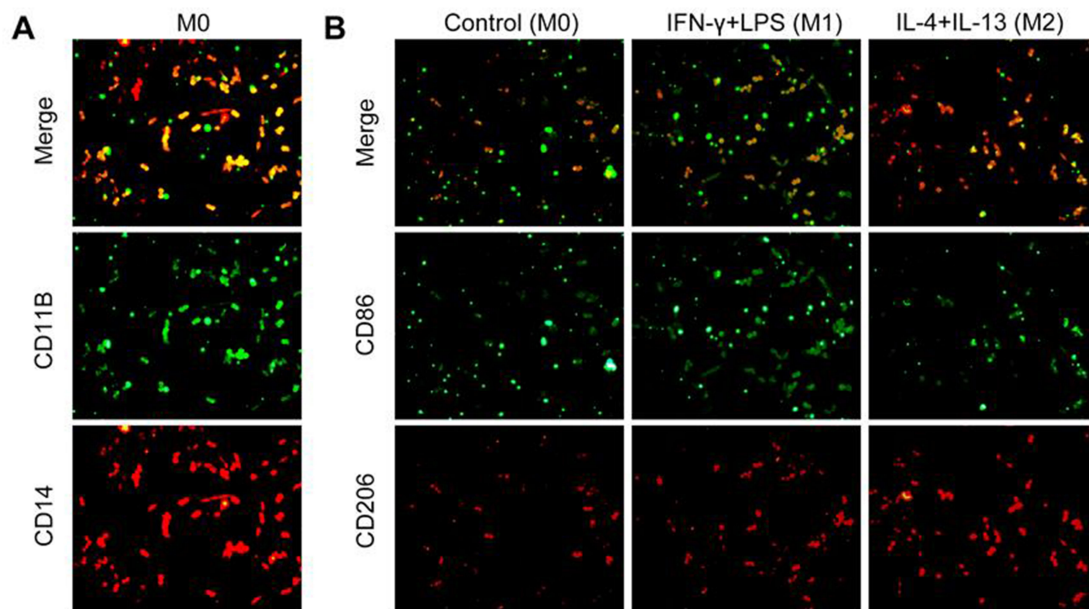


**Figure 1** (A) H&E staining was used to detect the pathological state of the adenoids of AH patients. (B) IHC based detection of the expressions of CD86, CD206, IL-17A and FOXP3.

expression of macrophage polarization and T lymphocyte infiltration markers CD86 and CD206, IL-17A and FOXP3 were detected by IHC. The outcomes exhibited that in comparison to the LE group, the expressions of M1 macrophage marker CD86 and IL-17 as a marker of Th17 cells were significantly increased, whereas, the expressions of M2 macrophage marker CD206 and FOXP3 expression as a marker of Treg cells were significantly decreased in the HE group (Figure 1B).

## CCL20/CCR6 Mediated Macrophage Polarization into M1-Type Cells so as to Promote the Differentiation of Lymphocytes into Th17 Cells in vitro in AH Lymphocytes

After the pro-inflammatory and alternative stimuli leading to cytokine-induced polarization of THP-1 cells, IF was used to detect the cell polarization status, and the results displayed that THP-1 cells were successfully polarized to M0, M1 and M2 macrophages (Figure 2A and B). The polarized M0, M1 and M2 macrophages were used to culture H9 cells, and the cells were grouped into control, M0, M1 and M2 groups. Initially, CCK-8 assay revealed no significant differences in cell survival rate between the control group and M0 group. Compared to M0, the cell survival rate of M1 group



**Figure 2** (A and B) Detection of polarization of macrophages by IF.

significantly increased 24 hr and 48 hr post-induction. The cell survival rate of M2 group increased after 48 hr of induction (Figure 3A).

The analysis of expressions of marker factors by IF involving the Th17 cells and Treg cells showed that in comparison to the M0 group, IL-17 expression in M1 group was significantly increased, while, FOXP3 expression was not significantly changed; however, IL-17 expression in M2 group was not significantly changed, but FOXP3 expression was significantly increased (Figure 3B). Supporting these outcomes, ELISA results showed that the trend of expression of IL-17 was compatible with or resembled the effects reported by IF. The expression results of Treg cell markers showed that in comparison with M0, the expression of IL-10 in M2 was significantly increased, whereas, no significant changes were observed in M1. Compared with M0, TGF- $\beta$  expression was significantly increased in both M1 and M2 (Figure 3C). Therefore, CCL20/CCR6 mediated macrophage polarization into M1-type can stimulate the differentiation of AH lymphocytes into Th17 cells.

## CCL20/CCR6 Mediated Macrophage Polarization into M1-Type Cells so as to Promote Adenoid Epithelial Inflammation in vitro in AH Cells

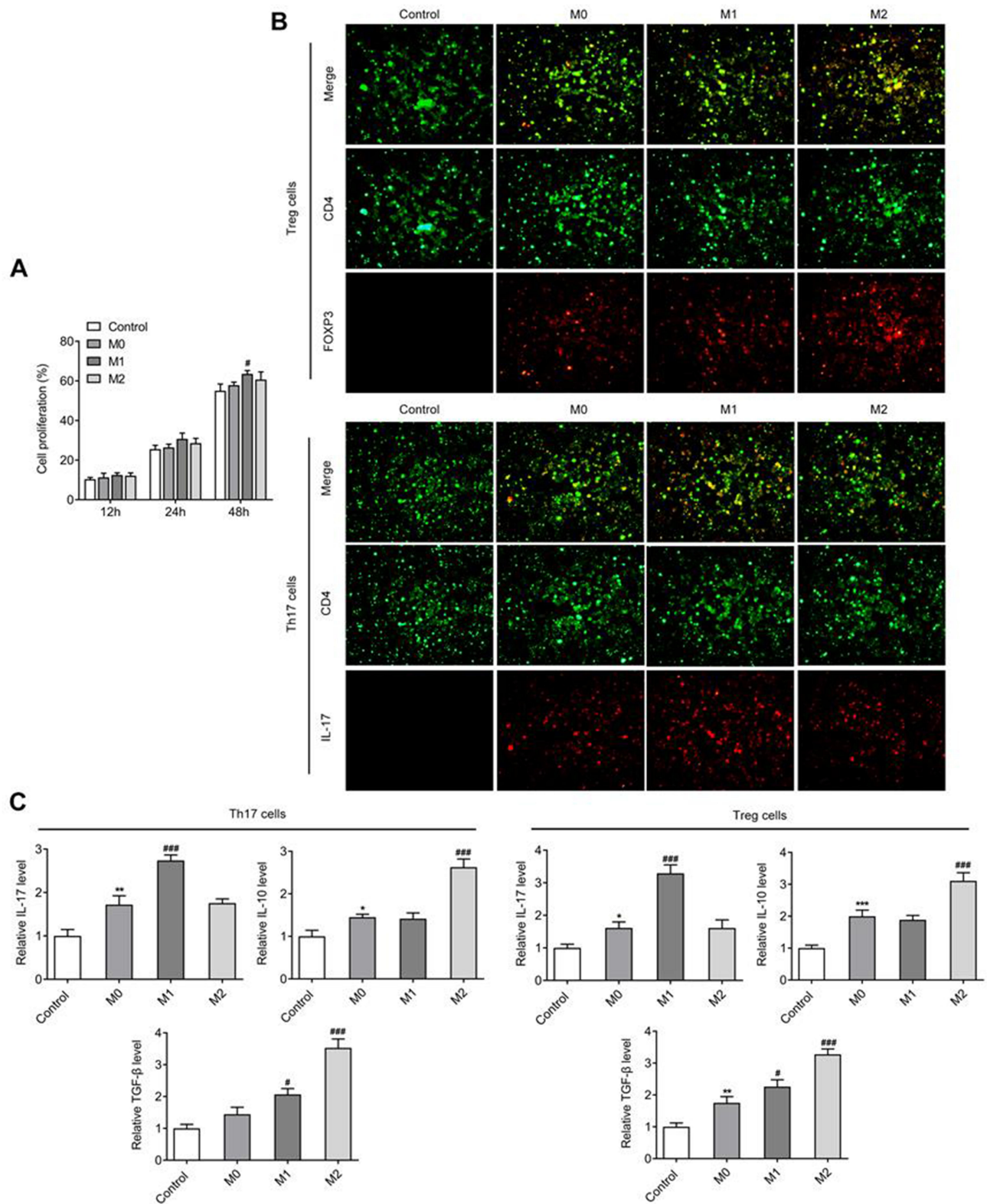
After identifying the roles of CCL20/CCR6 axis in polarization and differentiation of lymphocytes into Th17 cells, adenoid epithelial cells were cultured with polarized M0, M1, M2 macrophage medium, and treated with CCR6 inhibitor. The cells were grouped into control, M0, M1, M2 and M1+CCR6 inhibitor. CCK-8 results revealed that compared with M0, the cellular activities of M1 and M2 groups were significantly increased 24 hr and 48 hr after induction, and an increasing trend observed in the M1 group was more significant. Compared with the M1 group, cell activity was significantly decreased in M1+CCR6 inhibitor group (Figure 4A).

ELISA was used to distinguish the expressions of inflammatory cytokines and CCL20. The outcomes disclosed that compared to M0, the expressions of IL-6, TNF- $\alpha$ , IL-1 $\beta$  and CCL20 in M1 group were markedly greater, and the expressions of these cytokines in M2 group also showed an upward trend. But this upward trend was not as obvious as that of the M1. In addition, the expressions were significantly decreased in M2+CCR6 inhibitor group in comparison to the M2 group (Figure 4B). Nevertheless, the expression trend of CCR6 was consistent with that of CCL20 (Figure 5A). Western blot analysis of expressions of the inflammatory pathway proteins showed that compared with M0 group, the expressions of NLRP3, cleaved-caspase 1, and GSDMD-N were significantly increased in M1 group, but this was not obvious in M2 group. Compared with M1 group, expression of the components of inflammasome-induced apoptosis decreased significantly in M1+CCR6 inhibitor group (Figure 5B). Collectively, these results suggest that the CCL20/CCR6 mediated macrophage activation and polarization into M1-type macrophages can promote adenoid epithelial inflammation in AH.

## Discussion

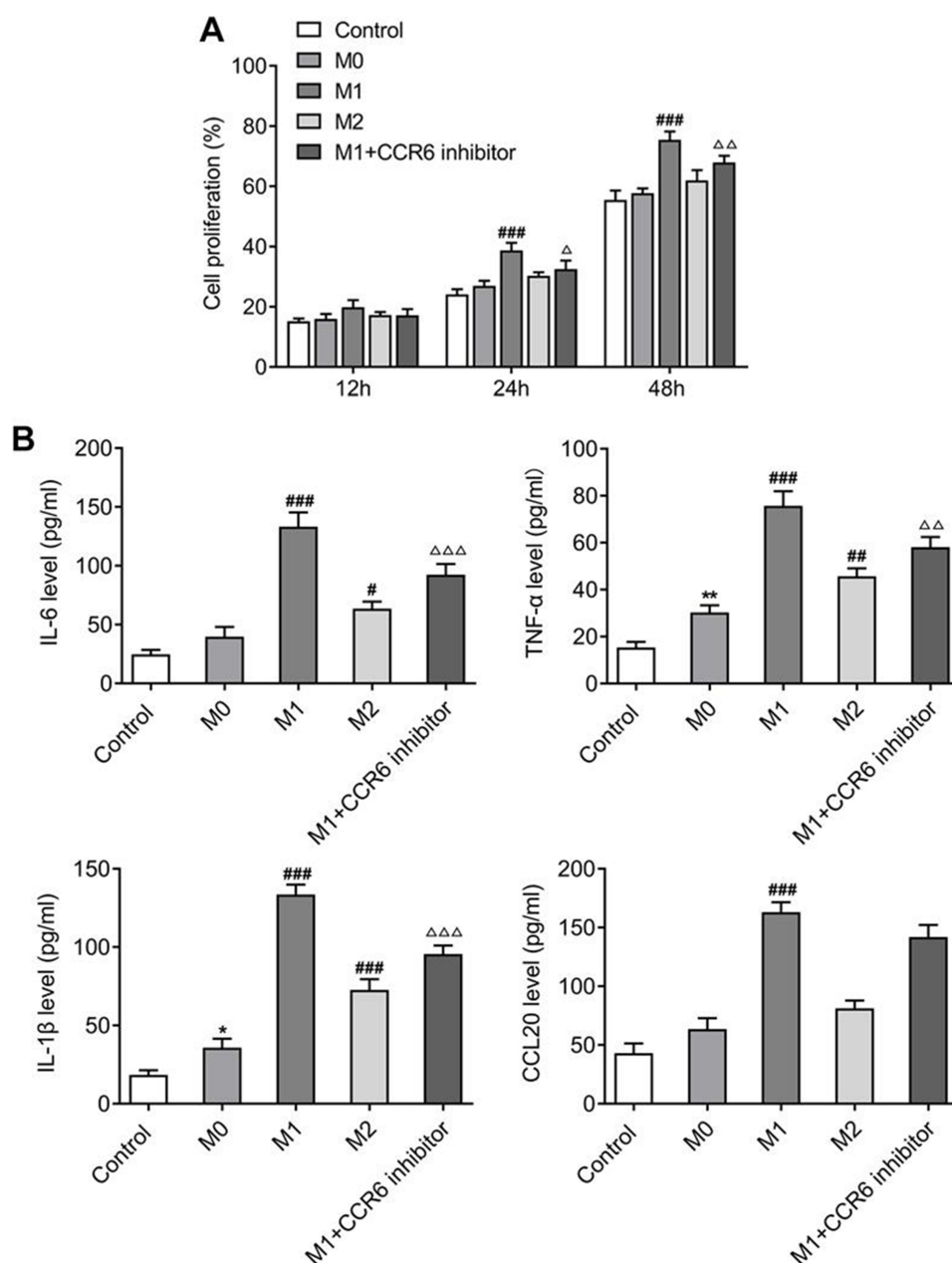
AH may be caused by bacteria, viruses or allergy. It is usually presented as the proliferation or increase in size of loose lymphoid connective tissues and their follicles or of the mucosal epithelial cells.<sup>24</sup> The proliferation process is also accompanied by a diverse group of immune cells enriched in the meantime or on the surface, further aggravating the stimulation. For the inflammatory hyperplasia of epithelial cells on the surface of the adenoid, the secreted products are more likely to invade forward to cause rhinosinusitis, and invade the eustachian tube on both sides to lead to otitis media.<sup>25</sup> In addition, previous research and published studies have found that the number of Th17 cells in the adenoid tissue or peripheral blood of AH patients is significantly increased, and Th17/Treg ratio is related to adenoid size, so the increase of Th17/Treg ratio is also an inducer of AH.<sup>23,26</sup> Therefore, in our experiment, the symptoms of AH were determined by the detection of adenoid epithelial cell inflammation and Th17/Treg ratio in T lymphocytes.

Macrophages have remarkable plasticity and are able to alter their functional phenotypes in response to the tissue microenvironment, thus performing a range of different functions necessary for host defense and tissue repair.<sup>27</sup> It has been acknowledged that there is a cooperative relationship between monocytes, macrophages and granulocytes in the adenoids to control the nasopharyngeal bacterial community as a whole.<sup>28</sup> Monocytes and macrophages produce inflammatory mediators such as leukotrienes that can promote the symptoms of AH.<sup>29</sup> Macrophages play a key



**Figure 3** (A) CCK-8 assay for identifying the proliferation of lymphocytes. (B) IF mediated detection of the expressions of CD4, IL-17 and FOXP3. (C) Detection of the levels of IL-17, IL-10 and TGF- $\beta$  by ELISA. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs control; # $P < 0.05$ , #### $P < 0.001$  vs M0. All experiments were performed in triplicate and the data are presented as mean  $\pm$  SD.



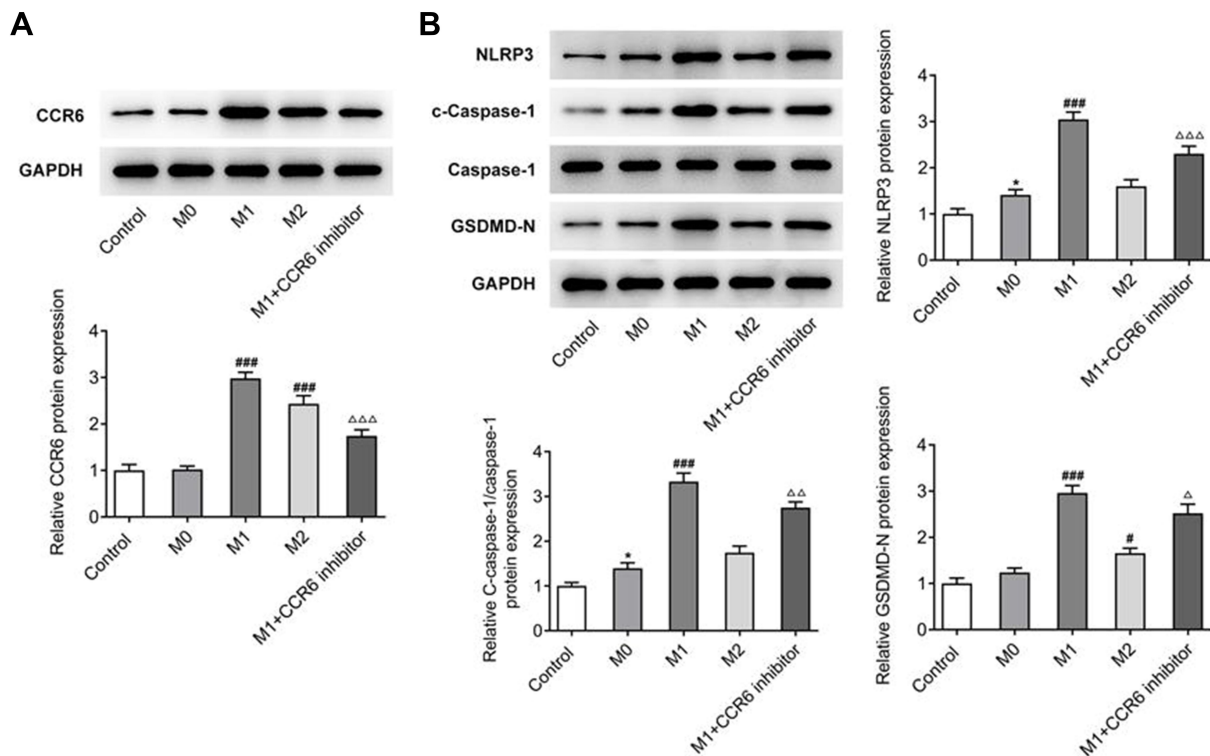


**Figure 4** (A) CCK-8 assay for identifying the proliferation of adenoid epithelial cells. (B) Detection of the levels of IL6, TNF- $\alpha$ , IL-1 $\beta$  and CCL20 by ELISA. \* $P < 0.05$ , \*\* $P < 0.01$  vs control; # $P < 0.05$ , ### $P < 0.01$ , #### $P < 0.001$  vs M0;  $\Delta P < 0.05$ ,  $\Delta\Delta P < 0.01$ ,  $\Delta\Delta\Delta P < 0.001$  vs M1. All experiments were performed in triplicate and the data are presented as mean  $\pm$  SD.

pathogenic role in AH instigated by bacterial infections and inflammations that arise during allergies.<sup>30</sup> Besides, immunological reactions and their responses play critical roles in AH physiology and the increased levels of cytokines and the macrophage markers are commonly observed in AH tissues.<sup>31</sup>

Certain members of aerobic gram-positive *Streptococcus*, *Staphylococcus* and *Enterococcus* genus and gram-negative organisms like *Moraxella catarrhalis* and *Haemophilus influenza* have been identified to be present in adenoid tissues of patients with AH.<sup>32,33</sup> Among these organisms, gram-positive pathogens produce lipoteichoic acid known to regulate macrophages and activate leukocytes to involve in creating an inflammatory reaction.<sup>34–38</sup> Also, the above-mentioned gram-negative pathogens can produce or constitute lipopolysaccharides.<sup>39,40</sup> Both lipoteichoic acid and lipopolysaccharides can activate monocytes/macrophages, act as M1 macrophage signals and generate an inflammatory response by causing a cytokine trigger.<sup>41–45</sup>





**Figure 5** (A) Detection of the expression of CCR6 by Western blot. (B) Detection of the expression of inflammation related proteins by Western blot. \* $P < 0.05$  vs control; <sup>#</sup> $P < 0.05$ , <sup>###</sup> $P < 0.001$  vs M0; <sup>Δ</sup> $P < 0.05$ , <sup>ΔΔ</sup> $P < 0.01$ , <sup>ΔΔΔ</sup> $P < 0.001$  vs M1. All experiments were performed in triplicate and the data are presented as mean  $\pm$  SD.

Hence, polarization of macrophages is an essential process for the formation of their phenotypes, namely, classically activated M1 and alternatively activated M2 macrophages specific to a stimulus. This process is necessary for tissue repair and maintaining homeostasis and the polarized macrophages possess unique surface markers and cytokines for a specified function.<sup>46</sup> Macrophages are classically activated for an M1 response by treatment with IFN- $\gamma$  and LPS and characterized by elevated expressions of CD86, whereas, the M2 response is alternatively activated by treatment with IL-4 and IL-13 and characterized by elevated expressions of CD206.<sup>47</sup> Typically, M1 macrophages demonstrate pro-inflammatory and antimicrobial properties, whereas, M2 macrophages possess anti-inflammatory properties.<sup>46</sup>

Therefore, infecting polarized macrophages with pathogenic microbes can result in the enhanced release of pro-inflammatory cytokines.<sup>48</sup> The cytokines IL-1 $\beta$ , IL-6, IL-17, IL-10, TNF- $\alpha$  and TGF- $\beta$  are involved in pro-inflammatory activities, resisting pathogens, pathologizing a number of inflammation-related disorders and enhancing the polarization of macrophages.<sup>49–59</sup> Also, FOXP3 and IL-17A are considered important Treg and Th17-related markers.<sup>60,61</sup> Adding to this, CCL20 is expressed by both lymphoid and non-lymphoid tissues to maintain homeostasis and immune functions. Under an array of conditions characterized by inflammation or hypoxia, cells infected by bacteria and viruses along with cells that release pro-inflammatory cytokines induce CCL20 production. Several cells that engage in inflammation including endothelial cells that form the lining of blood vessels, cells of the innate immune system like neutrophils and natural killer cells, and cells that mediate humoral immunity such as effector Th17 cells and B lymphocytes can secrete CCL20. Remarkably, dendritic cells, Langerhans cells and macrophages can also produce the chemokine.<sup>62–64</sup> TGF- $\beta$  regulates the expression of CCR6 in Th17 cells, and these pro-inflammatory cells are dependent on CCR6 for promoting the improved migratory properties and engagement of Th17 and Treg cells to the tissues at the site of inflammation.<sup>65</sup> In our study, we establish that in AH disease, macrophages can polarize into M1-type macrophages to promote lymphocyte differentiation, increase the Th17/Treg ratio and epithelial cell inflammation, ultimately promoting the development of AH disease.

CCL20 is the only known ligand with high affinity that binds CCR6 and drives the migration of CCR6+ cells in tissues.<sup>66</sup> It has been known that macrophages can secrete the chemokine CCL20 after activation, and CCL20 can bind to

receptor CCR6 in T lymphocytes to induce the lymphocytes to differentiate into Th17 cells and increase the Th17/Treg cell ratio.<sup>67,68</sup> Th17/Treg ratio is a critical factor in the pathogenesis of AH.<sup>26</sup> In addition, it has been reported that the expression of CCL20 is increased by multiple fold accredited to conditions that relate to inflammation, and the expression of CCR6 in such inflammatory diseases is strongly correlated with how severe the disease is.<sup>69</sup>

Hence, it was thought-provoking to guesstimate that there are some interesting relationships between CCL20/CCR6 axis and AH. To gain a basic understanding of how the CCL20-CCR6 axis interacts with AH and attains a relationship, we continued with our experiment and it was found that the expressions of CCL20 and CCR6 were significantly elevated in samples obtained from AH patients. The more obvious the AH was, the higher the expressions of CCL20 and CCR6 were. In addition, CCL20/CCR6 mediated lymphocyte differentiation and macrophage polarization can aggravate inflammation.<sup>70</sup> Likewise, the recruitment and participation of CCL20-CCR6 axis can promote the infiltration, activation and migration of macrophages.<sup>71,72</sup> In our experiment, we found that the inhibition of CCR6 expression could inhibit the expression of CCL20, thus reversing the promoting effect of M1-type macrophage polarization on epithelial cell inflammation.

NLRP3 inflammasome is brought together in responding to microbial encounters and infections. Gasdermin D (GSDMD) is a vital factor or constituent of inflammasomes and when it can activate macrophages, is recruited for cleaving by caspase-1 to form N-GSDMD. These fragments oligomerize across the plasma membrane, resulting in the formation of pores leading to improved permeability of membranes, mediating pyroptosis and engage in releasing IL-1 $\beta$ .<sup>73,74</sup> The upregulated expressions of these factors observed in this study indicate the roles of these components in the possible cell killing.

## Conclusion

To summarize, our research illustrates that CCL20/CCR6 mediated macrophage activation and polarization into M1-type macrophages can promote adenoid epithelial inflammation in AH.

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## Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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