

Suppressive effects of ketotifen on Th1- and Th2-related chemokines of monocytes

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Ketotifen is a mast cell stabilizer and useful in younger children with allergic diseases such as asthma and allergic rhinitis. Macrophage-derived chemokine (MDC) is a T-helper cell type 2 (Th2)-related chemokine involved in recruitment of Th2 cells toward allergen-challenged inflammation. However, the Th1-related chemokines, interferon-inducible protein 10 (IP-10) /CXCL10, and the monokine induced by interferon-gamma (MIG)/CXCL9 are also important in allergen-induced asthma in animal models. We investigated the effects of ketotifen on the expression of Th1- and Th2-related chemokines of human monocytes *in vitro* and *ex vivo*. Ketotifen (5–50 μ M) significantly down-regulated lipopolysaccharide (LPS)-induced MDC, MIG and IP-10 ($p < 0.05$, each comparison) in THP-1 cells and human primary monocytes in a dose-dependent manner. SB203580 [p38-mitogen-activated protein kinase (MAPK) inhibitor] suppressed LPS-induced MDC and IP-10 expression, and PD98059 (ERK–MAPK inhibitor) could only suppress LPS-induced IP-10, but not MDC expression. LPS-induced pp38 and p-ERK expression of THP-1 monocytic cells was suppressed by ketotifen. These data demonstrate that ketotifen is effective in down-regulating LPS-induced MDC, MIG and IP-10, which play important roles in the pathogenesis of airway inflammation. The suppressive effect on MDC and IP-10 may, at least in part, involve the down-regulation of LPS-induced p38 and ERK–MAPK expression.

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Asthma is a chronic inflammatory disorder (1). Infiltrating eosinophils, macrophages and monocytes comprise the largest population of cells within the airways of patients with allergic asthma and are associated with the induction of reversible airway hyper-reactivity (2). Evidence indicates that T-helper cell type 2 (Th2) immunoregulatory cytokines play a central role in the pathogenesis of airway inflammation in asthma (2). Macrophage-derived chemokine (MDC) is a Th2-related chemokine involved in recruitment of chemokine CC chemokine receptor 4-bearing Th2 cells in allergen-challenged inflammation (3, 4). The Th1-related chemokines, interferon-inducible protein 10 (IP-10) /CXCL10 and the

monokine induced by interferon-gamma (MIG) /CXCL9, are also important chemokines during the human allergic pulmonary reaction (5, 6).

The oral medications for maintenance therapy of asthma or allergic diseases in children include theophylline, leukotriene antagonists and ketotifen. Ketotifen is also used successfully to prevent the development of asthma in pre-asthmatic children (7–9). Our previous data showed that ketotifen, but not montelukast (a leukotriene receptor antagonist), suppressed plasma MDC and thymus and activation-regulated chemokine (TARC) concentrations in asthmatic children (10). However, there are still no data on the influence of ketotifen on Th1- and Th2-related

chemokine expression of monocytes *in vitro* and *ex vivo*. Exposure to airborne endotoxin [lipopolysaccharide (LPS)] in infancy may protect against asthma by promoting enhanced Th1 response and tolerance to allergens. However, later in life, it adversely affects patients with asthma (11). We investigated whether ketotifen could suppress the LPS-induced MDC, MIG and IP-10 expression of monocytes, and explored the detailed mechanisms about the effects of ketotifen on LPS-induced chemokines expression of monocytes that may involve in the mechanisms of allergic diseases or asthma.

Methods

Cell preparation

The human monocytic cell line THP-1 (American Type Culture Collection, Rockville, MD, USA) was cultured in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% foetal bovine serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin at 37°C and 5% CO₂ in a humidified incubator. Cells were centrifuged and resuspended in fresh media in 24-well plates at a concentration of 10⁶/ml for 24 h before experimental use. The cells were pre-treated with ketotifen 2 h before LPS (0.2 µg/ml) (*Escherichia coli*; Sigma Chemical Co.) stimulation. Cell supernatant was collected at 12, 24 and 48 h after LPS stimulation.

Peripheral blood samples were from healthy individuals who had no personal or family history of allergies (n = 3). Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation (Lymphoprep, Oslo, Norway). Human primary monocytes were isolated from PBMCs by magnetic bead sorting with anti-CD14 monoclonal antibody (MACS, Miltenyi Biotec, Germany).

ELISA assay

The MDC, MIG, IP-10 and IL-8 concentrations of cell supernatants were determined using commercially available enzyme-linked immunosorbent assay (ELISA)-based assay systems (R&D System, Minneapolis, MN, USA). Assays were performed using the protocols recommended by the manufacturer.

Reverse transcriptase polymerase chain reaction

THP-1 cells were pre-treated with ketotifen (1–10 µM) 2 h before LPS (0.2 µg/ml) stimulation. Total RNA extraction from the cells was performed 6 h after LPS stimulation with Trizol

(Life Technologies, Inc., Eggenstein, Germany), according to the acid guanidium thiocyanate-phenol-chloroform method. cDNA was synthesized with random priming from 5 µg of total RNA. The polymerase chain reaction (PCR) reaction was performed in a Perkin-Elmer thermalcycler (Norwalk, CT, USA) with the primers used for the amplification of MDC. The amplification consisted of denaturation at 94°C for 45 s, annealing at 56°C for 45 s and extension at 72°C for 45 s (33 cycles). A total of 10 µl of PCR products were separated onto 2% w/v agarose gels and stained with ethidium bromide.

Mitogen-activated protein kinase assay

After 1 h of treatment with or without three mitogen-activated protein kinase (MAPK) inhibitors (PD 98059, SB203580 and SP600125), the cells were stimulated with LPS (0.2 µg/ml). Cell supernatant was collected 48 h after LPS stimulation for ELISA assay.

Western blotting

After treatment for 2 h with or without ketotifen (1–10 µM), the cells were stimulated with

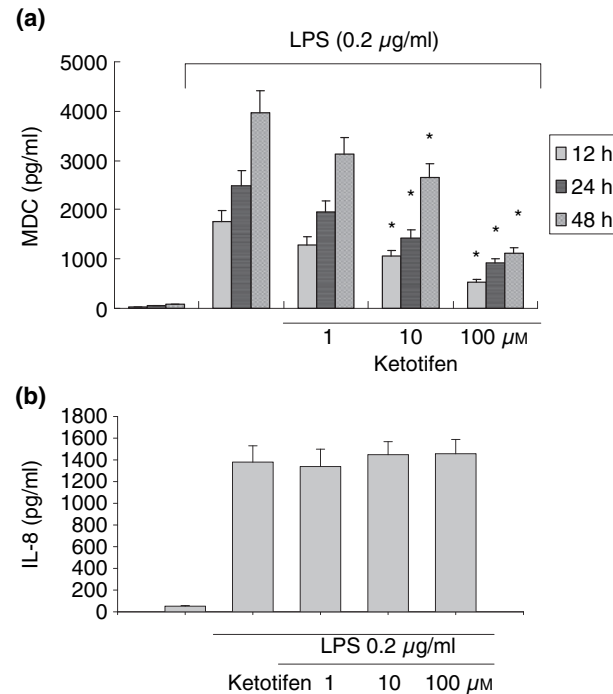


Fig. 1. Ketotifen suppressed the lipopolysaccharide (LPS)-induced macrophage-derived chemokine (MDC) production of THP-1 cells after 12 h, 24 h and 48 h (a) of LPS stimulation, but did not suppress interleukin (IL)-8 production (24 h) (b) (*p < 0.05 between LPS only and LPS plus ketotifen treatment).

LPS (0.2 $\mu\text{g/ml}$) and lysed with equal volumes of ice-cold 150- μl lysis buffer 1 h later. After centrifugation at $13,000 \times g$ for 15 min, equal amounts of cell lysates were analysed by Western blot with anti-MAPK (p38, ERK and JNK) and anti-phospho-MAPK (pp38, p-ERK and p-JNK) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Boston, MA, USA).

Statistical analyses

All data are presented as mean $\pm$ standard deviation. One-way analysis of variance was used for all statistical comparisons, and the Student-Newman-Keuls test was conducted for multiple comparisons. A p-value of <0.05 was considered indicative of significant between-group differences. SigmaStat software (Jandel

Scientific, Erkrath, Germany) was used for all statistical analyses.

Results

Ketotifen, but not leukotriene receptor antagonists, suppressed LPS-induced MDC expression of THP-1 and human primary monocytes

Ketotifen (10–100 μM) significantly down-regulated LPS-induced MDC production of THP-1 cells after LPS stimulation for 12, 24 and 48 h (Fig. 1a), but did not significantly suppress IL-8 production (Fig. 1b). Montelukast and zafirlukast did not reduce the LPS-induced MDC production of THP-1 cells (Fig. 2a, b) after 12 h of LPS stimulation. Ketotifen (1–10 μM) significantly reduced LPS-induced MDC mRNA expression of THP-1 cells after LPS stimulation (Fig. 3a). Ketotifen (0.5–50 μM) also significantly suppressed LPS-induced MDC production of human primary monocytes (Fig. 3b).

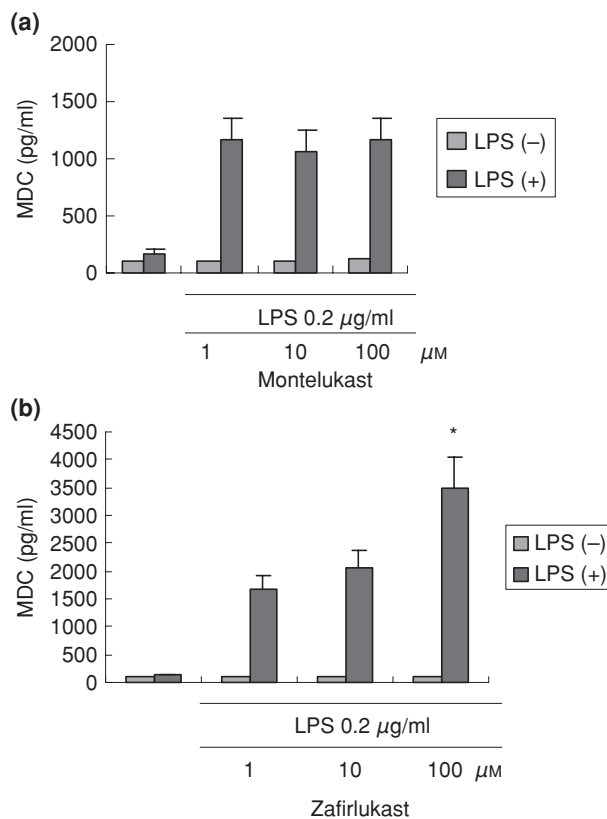


Fig. 2. Montelukast and zafirlukast did not reduce the lipopolysaccharide (LPS)-induced macrophage-derived chemokine (MDC) production of THP-1 cells (Fig. 2a, b) after 12 h of LPS stimulation (* $p < 0.05$ between LPS only and LPS plus zafirlukast treatment).

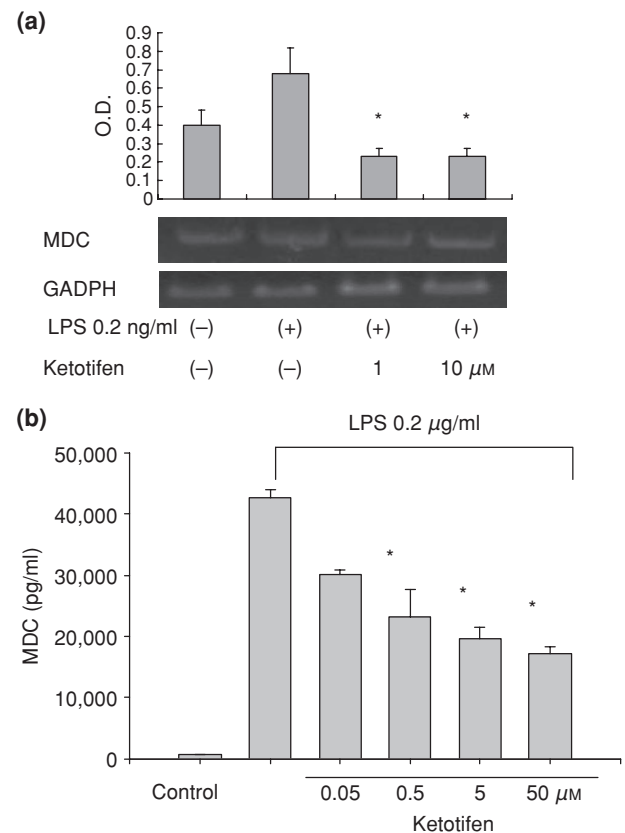


Fig. 3. Ketotifen (1–10 μM) significantly reduced lipopolysaccharide (LPS)-induced macrophage-derived chemokine (MDC) mRNA expression of THP-1 cells after LPS stimulation (Fig. 3a). Ketotifen (0.5–50 μM) also significantly suppressed LPS-induced MDC production of human primary monocytes (Fig. 3b) (* $p < 0.05$ between LPS only and LPS plus ketotifen treatment).

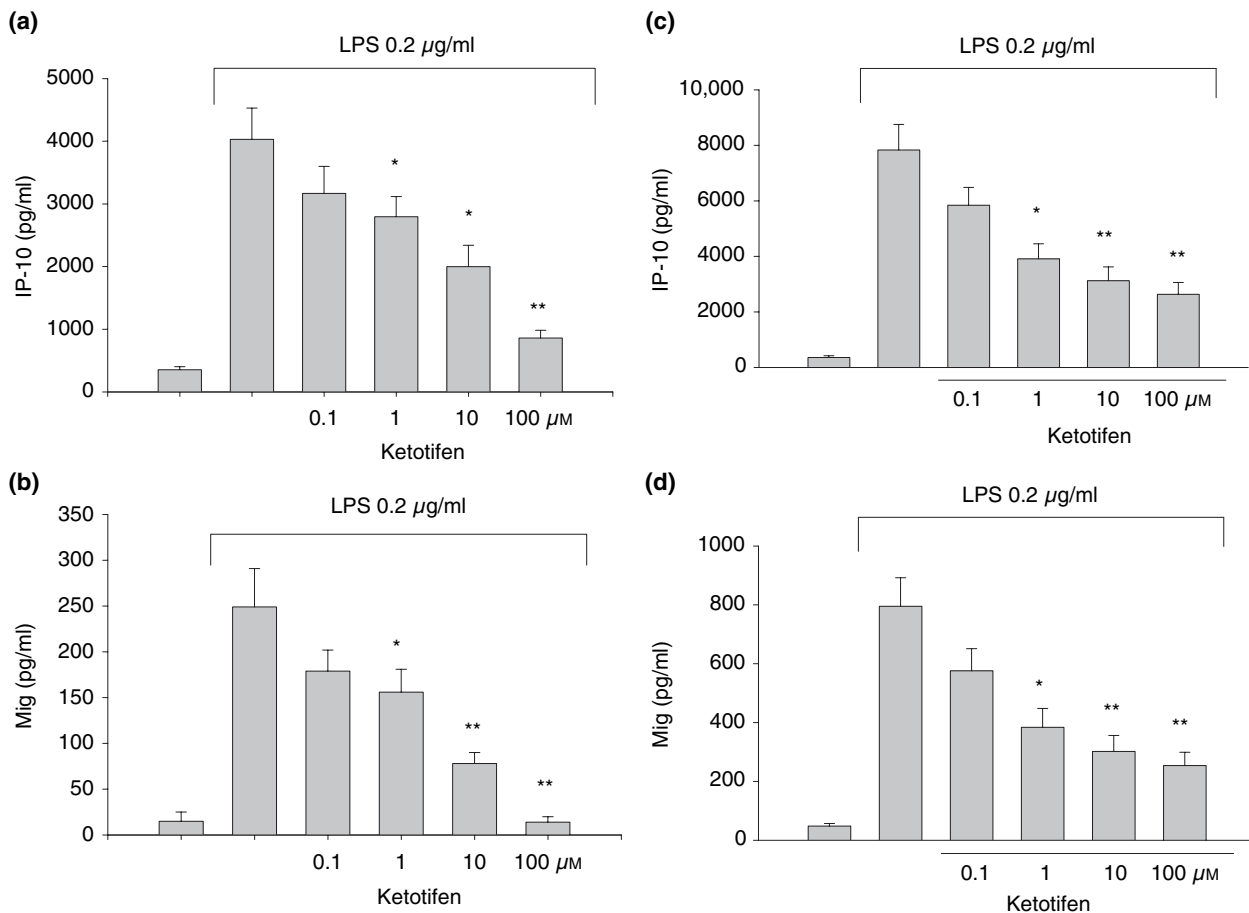


Fig. 4. Ketotifen suppressed the lipopolysaccharide (LPS)-induced interferon-inducible protein-10 (IP-10) (a) and monokine induced by interferon-gamma (MIG) (b) production of THP-1 cells after 24 h of LPS stimulation. Ketotifen also suppressed the LPS-induced IP-10 (c) and MIG (d) production of human primary monocytes after 24 h of LPS stimulation (* $p < 0.05$ and ** $p < 0.01$ between LPS only and LPS plus ketotifen treatment).

Ketotifen suppressed LPS-induced IP-10 and MIG expression of THP-1 cells and human primary monocytes

Ketotifen (1–100 μM) significantly down-regulated LPS-induced IP-10 (Fig. 4a) and MIG (Fig. 4b) production of THP-1 cells after LPS stimulation. Ketotifen (1–100 μM) significantly down-regulated LPS-induced IP-10 (Fig. 4c) and MIG (Fig. 4d) production of human primary monocytes after LPS stimulation.

Ketotifen suppressed LPS-induced pp38 expression of THP-1 cells

Fig. 5a, b shows that SB203580 (p38-MAPK inhibitor) suppressed LPS-induced MDC and IP-10 expression, suggesting that p38-MAPK signalling is an important factor of LPS-induced MDC and IP-10 expression of THP-1 cells. SB203580 and SP600125 suppressed LPS-induced MDC and IP-10 expression. PD98059 could only suppress LPS-induced IP-10, but not

MDC expression. Pre-treatment with ketotifen (1–10 μM) for 2 h significantly suppressed LPS-induced pp38 MAPK expression on THP-1 cells ($p < 0.05$) (Fig. 5c). Ketotifen (1–10 μM) could suppress LPS-induced p-ERK, but not p-JNK expression of THP-1 cells (Fig. 5d).

Discussion

Ketotifen shares properties with anti-histamines and mast cell stabilizers (12). Clinical studies have shown ketotifen to be effective in the treatment of allergic diseases such as asthma and allergic rhinitis. In our previous study, ketotifen controlled the symptoms of asthma and suppressed plasma MDC and TARC concentrations in asthmatic children (7). Plasma MDC and TARC concentrations are increased and negative correlated with peak expiratory flow rate percentage of predicted value in children with acute asthmatic attack (13). MDC in

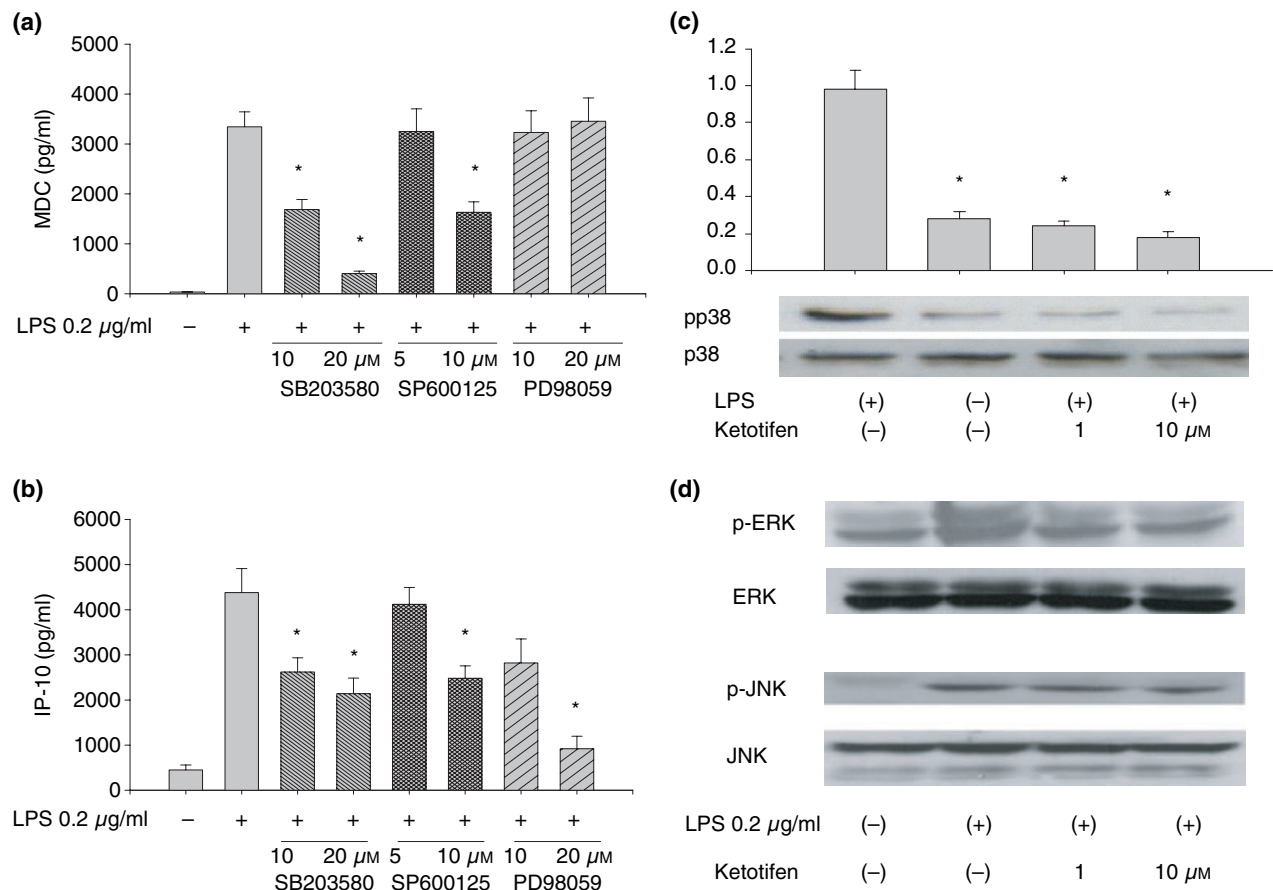


Fig. 5. SB203580 and SP600125 suppressed lipopolysaccharide (LPS)-induced macrophage-derived chemokine (MDC) (a) and interferon-inducible protein-10 (IP-10) (b) expression. PD98059 could only suppress LPS-induced IP-10 expression. LPS-induced pp38 expression of THP-1 cells was suppressed by ketotifen (c). Ketotifen could reduce LPS-induced p-ERK, but not p-JNK expression of THP-1 cells (d).

exhaled breath condensate (EBC) and MDC and TARC in plasma are increased in children with persistent asthma as compared with that in intermittent asthma or control children (14). MDC concentrations in EBC are suppressed in patients with high-dose inhaled corticosteroid treatment (14). MDC, TARC, IP-10 and MIG are significantly increased in bronchial alveolar fluid in asthmatic children (15). However, the effect of ketotifen on allergy-related chemokines has not been reported *in vitro*. Chemokines play an important role in the pathophysiology of asthma and allergy. Recent studies showed that bronchial epithelium in asthmatic patients expressed TARC and MDC, which are both implicated in the recruitment of Th2 cells toward inflammatory airways (3).

Exposure to airborne endotoxin in infancy may protect against asthma by promoting enhanced Th1 response and tolerance to allergens. However, later in life, it adversely affects patients with asthma (11). Airborne endotoxin

adversely affects patients with asthma in three ways: increasing the severity of the airway inflammation; increasing the susceptibility to rhinovirus-induced colds; and causing chronic bronchitis and emphysema with the development of irreversible airway obstruction after chronic exposure in adults (11). Endotoxin can increase disease severity by acting as a natural adjuvant to augment asthma and atopic inflammation. Recent studies suggest that it can even act on its own, causing a distinct endotoxic form of asthma (16). In this present study, MDC was highly elevated in LPS-stimulated monocytes, which may also explain how LPS adversely affects patients with asthma.

Our results clearly show a significant inhibitory effect of ketotifen on MDC, IP-10 and MIG expression, which is related to the recruitment of Th2 and Th1 cells. Previous research has shown that ketotifen inhibits chemotaxis of eosinophils (17). These observations suggest that the therapeutic effect of ketotifen on allergic diseases

might limit the recruitment of Th2, Th1 cells and eosinophils. However, ketotifen did not suppress IL-8 expression, which is related to the chemotaxis of neutrophils. In severe asthma, epithelial damage has the potential to contribute to neutrophilic inflammation through enhanced production of IL-8 (18). Therefore, ketotifen may not be indicated for severe asthmatic patients.

Asthma is a complicated inflammatory disorder. Although Th2-type immunoregulatory cytokines play a central role in the pathogenesis of airway inflammation in asthma, the Th1-related chemokine IP-10 is also important during the human allergic pulmonary late-phase reaction (5). Overexpression of IP-10 in transgenic mice increased airway hyper-responsiveness, eosinophilia and IL-4 in an IFN γ -independent fashion (19). Both Th2 and Th1 reactions are involved in asthmatic airway inflammation. The effects of ketotifen on allergic diseases and asthma may at least, in part, through reduction of Th1- and Th2- related chemokines.

Some reports showed leukotriene receptor antagonists could induce Churg–Strauss syndrome (20, 21). In the present study, zafirlukast could enhance the LPS-induced MDC production at higher dose. MDC is an important chemokine for Th2 cell recruitment. These results may explain partly that patients with Churg–Strauss syndrome also show elevated Th2-related cytokines, such as IL-4 and IL-13 production (22, 23). However, it is still difficult to say that these data have any relationship with the elevated liver enzymes in some patients receiving treatment of zafirlukast (24).

Th2 type immunoregulations play a central role in the pathogenesis of airway inflammatory in asthma. In the present study, ketotifen appeared to be more potent in suppressing the production of Th1-associated chemokines than that of Th2-associated chemokine MDC in THP-1 cells. MAPKs may involve in LPS-induced chemokines and cytokines expression of monocytes (25, 26). Suppressive effect of SB203580 and SP600125 on the expression of LPS-induced MDC and IP-10 of THP-1 cells suggested activation of p38 and JNK may be related with part of Th1- and Th2-chemokines production following LPS treatment of monocytes. ERK may only involve in IP-10, but not MDC expression because PD98059 could only decrease the IP-10, but not MDC production of THP-1 cells. Our data showed the inhibition of p38 activation by ketotifen may at least in part involve in the suppressive effects of ketotifen on the chemokines expression of LPS-stimulated monocytes (Fig. 5c). Ketotifen could suppress ERK, but

not JNK expression (Fig. 5d) that may explain why ketotifen appeared to be more potent in suppressing the production of IP-10 than that of MDC in THP-1 cells. Except the property of anti-histamines, ketotifen may have some anti-allergic effects via the suppressions on Th1- and Th2-related chemokines expression of monocytes.

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