

Pranlukast inhibits NF- κ B activation in human monocytes/macrophages and T cells

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Summary

Background Pranlukast is a leukotriene 1 (LT1) receptor antagonist and is effective against bronchial asthma. Pranlukast inhibits contraction of the tracheal muscle, and thereby antagonizes the binding of LTC₄, LTD₄ and LTE₄. However, the action of pranlukast on monocytes/macrophages and T cells is unknown.

Objective We examined whether or not pranlukast inhibits TNF- α -induced activation of nuclear transcription factor NF- κ B, a factor that is essential for the expression of proinflammatory cytokines, on human monocytic 1.3% dimethylsulphoxide (DMSO)-differentiated U-937 cells, which have cysteinyl LT1 (CysLT1) receptors on their membranes, and T cells (Jurkat), which do not.

Methods We examined whether or not LTC₄, LTD₄ or LTE₄ induced NF- κ B activation in 1.3% DMSO-differentiated U-937 cells by Western blotting. The inhibitory effects of pranlukast and MK-571, which is an LTD₄ receptor-selective antagonist, on TNF- α -induced NF- κ B activation was evaluated by Western blotting and flow cytometry, and those on lipopolysaccharide (LPS)-induced interleukin-6 (IL-6) production in peripheral blood mononuclear cells (PBMC) were evaluated by enzyme-linked immunosorbent assaying.

Results LTC₄, LTD₄ or LTE₄ did not induce NF- κ B activation in 1.3% DMSO-differentiated U-937 cells. Western blotting demonstrated that 10⁻⁵ M pranlukast inhibits NF- κ B activation in 1.3% DMSO-differentiated U-937 and Jurkat cells by about 40% & 30%, respectively. Flow cytometry demonstrated that pranlukast and MK-571 inhibit NF- κ B activation in 1.3% DMSO-differentiated U-937 and Jurkat cells in a dose-related manner. Moreover, 10⁻⁵ M pranlukast and MK-571 inhibited LPS-induced IL-6 production in PBMC by about 65% and 15%, respectively.

Conclusion Pranlukast and MK-571 partially inhibited NF- κ B activation in 1.3% DMSO-differentiated U-937 and Jurkat cells, and IL-6 release in PBMC. These findings are consistent with the idea that, independently of CysLT1 receptor antagonism, micromolar concentrations of pranlukast suppress the production of proinflammatory cytokines via inhibition of NF- κ B activation in monocytes/macrophages and T cells, but the contribution of this effect to the anti-inflammatory activity of pranlukast at oral therapeutic doses in asthmatic patients is unclear.

Keywords inflammation, Jurkat cell, MK-571, NF- κ B, peripheral blood mononuclear cell, pranlukast, U-937 cell

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Introduction

Pranlukast antagonizes the cysteinyl leukotriene 1 (CysLT1) receptor, and inhibits contraction induced by leukotriene C₄ (LTC₄), D₄ or E₄ [1]. Pranlukast has some other actions in asthma than as an antagonist of the CysLT1 receptor, including immunomodulation [2]. NF- κ B is a ubiquitous transcription factor for genes that encode proinflammatory cytokines such as IL-1, IL-6, IL-8 and TNF- α [3–7]. The prototype of NF- κ B is a heterodimer consisting of p50 and p65 bound by members of the I κ B family, including I κ B α , in the cytoplasm [8, 9].

Phosphorylation of I κ B by bacterial products, viruses, drugs and cytokines rapidly leads to I κ B degradation and translocation of NF- κ B to the nucleus [10, 11]. Activation of NF- κ B results in binding of specific promoter elements and expression of mRNAs for proinflammatory cytokine genes [3–7].

Bacterial and viral infections often induce bronchial asthma attacks, especially in children. It is important to modulate infectious inflammation on treatment of patients with bronchial asthma. Monocytes/macrophages and T cells play an essential role in the infectious inflammatory response, while the role of monocytes/macrophages and T cells in allergic inflammation remains unclear. Therefore, we tested the hypothesis that pranlukast modulates inflammation by inhibiting NF- κ B activation through experiments on human monocytic 1.3% dimethylsulphoxide (DMSO)-differentiated U-937 cells, which have CysLT1 receptors on their membranes [12], and T cells (Jurkat), which do not [13], stimulated by TNF- α .

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Materials and methods

Cell culture and stimulation conditions

U-937 cells, a human monocytic leukaemia cell line, and Jurkat cells, a human T cell leukaemia cell line, obtained from the American Type Culture Collection, were maintained at 37 °C under humidified 5% CO₂ as stationary cultures. The cells were grown in RPMI 1640 medium containing 10% FBS, 100 units/mL of penicillin, and 100 µg/mL of streptomycin. U-937 cells were allowed to differentiate for 4 to 5 days in the presence of 1.3% (v/v) DMSO.

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood from eight healthy medication-free volunteers, with informed consent, by Histopaque 1077 (Sigma Chemical Co., St Louis, MO, USA) gradient centrifugation, and then resuspended in RPMI 1640 medium containing 10% FBS, 100 units/mL of penicillin, and 100 µg/mL of streptomycin.

Cells were exposed to TNF- α (R & D Systems, Minneapolis, MN, USA), LTC₄, -D₄, -E₄ (Sigma Chemical Company), or lipopolysaccharide (LPS, from *Escherichia coli*, 0111:B4; Sigma Chemical Company) with or without pre-treatment with 10⁻⁷, 10⁻⁶ or 10⁻⁵ M pranlukast provided by ONO Pharmaceutical Co. (Osaka, Japan), MK-571, which is an LTD₄ receptor-selective antagonist (Alexis Biochemicals, San Diego, CA, USA), or disodium cromoglicate (DSCG) provided by Fujisawa Pharmaceutical Co. (Osaka, Japan) 30 min before incubation at 37 °C for the times indicated.

CysLT₁ receptors

DMSO-differentiated U-937 and Jurkat cells were labelled with a rabbit anti-CysLT₁ receptor polyclonal antibody (Cayman, Ann Arbor, MI, USA), and then labelled with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG monoclonal antibody (Sigma). Images were acquired, processed and quantified with an OLYMPUS BX50 microscope (OLYMPUS, Tokyo, Japan), a Micromac CCD camera (Princeton Instruments, San Diego, CA, USA), and the Metamorph imaging program (Universal Imaging, West Chester, PA, USA).

Nuclear extracts and Western blot analysis

Nuclear extracts were harvested from 1.3% DMSO-differentiated U-937 and Jurkat cells by the previously published procedures [14, 15]. The protein concentrations of the nuclear extracts were determined using Bio-Rad (Hercules, CA, USA) protein concentration reagent. Samples were stored at -80 °C. To determine the NF- κ B-p65 levels, samples containing 15 µg of protein were separated in denaturing 10% polyacrylamide gels and then transferred to polyvinylidene difluoride membranes. After three washes in TBST (40 mM Tris-HCl, pH 7.6, 300 mM NaCl, and 0.5% Tween 20), the membranes were incubated with 1:1000 diluted rabbit polyclonal anti-NF- κ B-p65 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBST containing 5% non-fat dry milk at room temperature for 1 h. After three washes in TBST, the membranes were incubated with 1:2500 diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) for 1 h at room temperature. Immunoreactive proteins were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA) and

analysed by radiography. Quantification of bands was conducted with a Kodak Digital Science 1D (Eastman Kodak Company, New Haven, CT, USA).

Flow cytometric analysis

Flow cytometric analysis was performed by the previously published procedure [16, 17]. The cells were permeabilized in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, containing 0.1% saponin and 10 mM HEPES. The cells were then labelled with a mouse anti-NF- κ B (nuclear-localized signal) antibody (IgG3; Boehringer Mannheim, Mannheim, Germany). The mouse anti-NF- κ B (nuclear-localized signal) antibody recognizes an epitope overlapping the nuclear location signal of NF- κ B-p65 and therefore selectively recognizes the activated form of NF- κ B. The cells were then labelled with a FITC-conjugated rat anti-mouse IgG3 monoclonal antibody (Pharmingen, San Diego, CA, USA). After washing, the cells were fixed with 1% paraformaldehyde in PBS and then stored at 4 °C until flow cytometric analysis. Single-colour immunofluorescence analysis of 5000 cells was performed with a FACScan flow cytometer equipped with CellQuest software (Becton-Dickinson Biosciences, San Jose, CA, USA).

Determination of IL-6 concentrations

PBMC were seeded into six-well tissue culture dishes at the density of 1 × 10⁶ cells/well and then exposed to 1 µg/mL lipopolysaccharide (LPS, from *Escherichia coli*, 0111:B4; Sigma) with or without pre-treatment with pranlukast, MK-571 or DSCG for 30 min. The culture fluids were collected 2 h after LPS addition. The concentrations of IL-6 in samples were determined with a sandwich-type enzyme-linked immunosorbent assay (ELISA) kit (R & D Systems). In our study, this assay had a detection limit of 3.1 pg/10⁶ cells.

Statistical analysis

Statistical analysis was performed with the Wilcoxon matched paired test, and a *P*-value of less than 0.05 was taken as significant.

Results

As can be seen in Fig. 1, 1.3% DMSO-differentiated U-937 cells had CysLT₁ receptors on their membranes, but Jurkat cells did not. Western blotting revealed that TNF- α activated NF- κ B in 1.3% DMSO-differentiated U-937 cells, but pranlukast, LTC₄, -D₄ and -E₄ did not (Fig. 2a, b). Pranlukast (10⁻⁵ M) inhibited NF- κ B activation in 1.3% DMSO-differentiated U-937 and Jurkat cells stimulated with TNF- α , but DSCG did not (Figs 3a, b, 4a, b). Flow cytometry of 1.3% DMSO-differentiated U-937 and Jurkat cells incubated with TNF- α demonstrated that pranlukast inhibited NF- κ B activation (Figs 5 & 6). The effect of pranlukast was dose-related. The effect of MK-571 was weaker than that of pranlukast. DSCG did not inhibit NF- κ B activation in differentiated U-937 or Jurkat cells (data not shown).

The concentrations of IL-6 in culture fluid of PBMC exposed to LPS in the presence and absence of DSCG, pranlukast or MK-571 for 2 h are shown in Fig. 7. The IL-6 concentration in culture fluid of cells exposed to only LPS was 64.6 ± 6.0 pg/10⁶

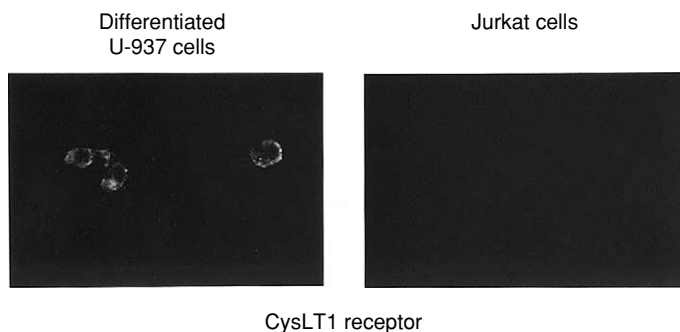


Fig. 1. Immunofluorescence microscopic image showing 1.3% DMSO-differentiated U-937 and Jurkat cells. Cells were labelled with anti-CysLT1 receptor antibodies and FITC-conjugated secondary antibodies.

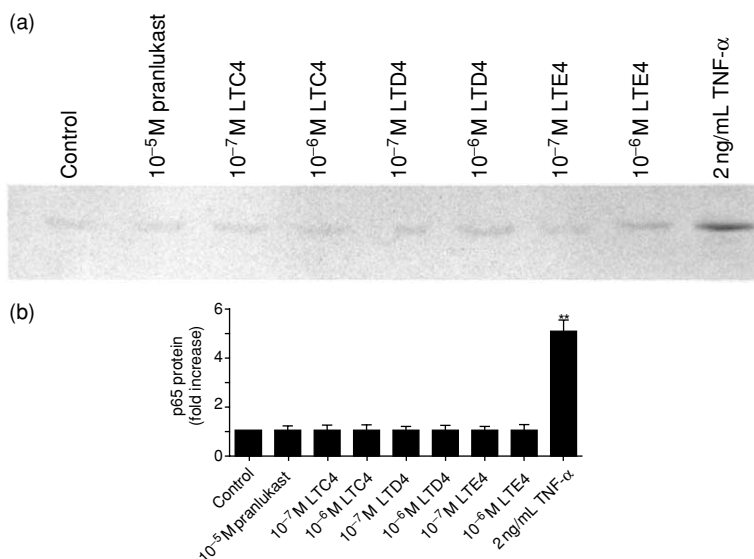


Fig. 2. Pranlukast, LTC4, LTD4, LTE4, or TNF- α -induced NF- κ B activation, as measured by Western blotting, in 1.3% DMSO-differentiated U-937 cells. Cells were exposed by pranlukast, LTC4, LTD4, LTE4 or TNF- α for 2 h. A nuclear extract was harvested for polyacrylamide gel electrophoresis and immunoblotted for p65. Representative blots are shown (a). After densitometric analysis, data ($n = 8$) were expressed as fold increase compared with untreated cells and presented as means $\pm$ SD (b). ** $P \leq 0.01$ compared with untreated cells.

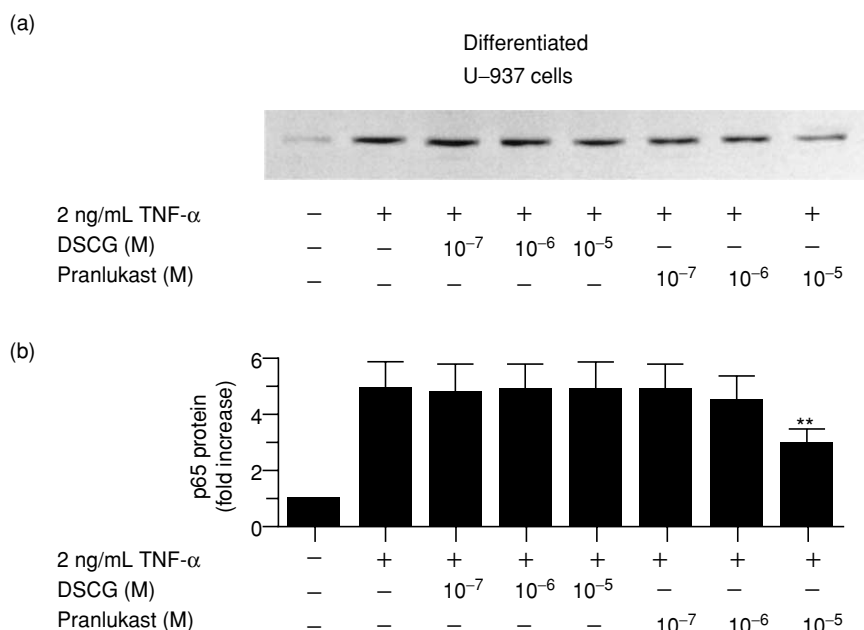
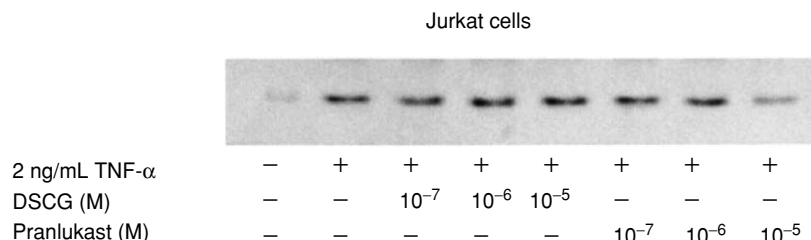


Fig. 3. Inhibitory effect of pranlukast or DSCG on NF- κ B activation, as measured by Western blotting, in 1.3% DMSO-differentiated U-937 cells stimulated with TNF- α for 2 h, and pre-treated with pranlukast or DSCG for 30 min before the TNF- α treatment. A nuclear extract was harvested for polyacrylamide gel electrophoresis and immunoblotted for p65. Representative blots are shown (a). After densitometric analysis, data ($n = 8$) were expressed as fold increase compared with untreated cells and presented as means $\pm$ SD (b). ** $P \leq 0.01$ compared with cells treated with TNF- α only.

(a)



(b)

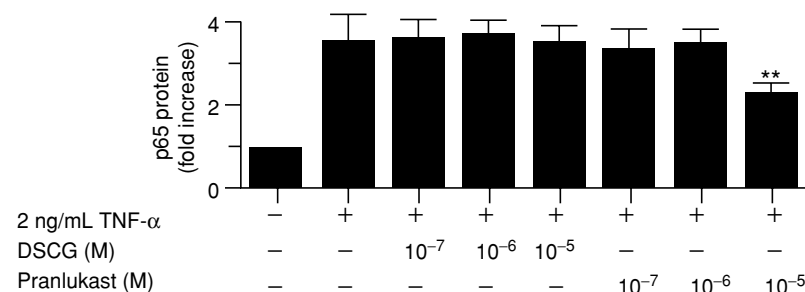


Fig. 4. Inhibitory effect of pranlukast or DSCG on NF- κ B activation, as measured by Western blotting, in Jurkat cells stimulated with TNF- α for 2 h, and pre-treated with pranlukast or DSCG for 30 min before the TNF- α treatment. A nuclear extract was harvested for polyacrylamide gel electrophoresis and immunoblotted for p65. Representative blots are shown (a). After densitometric analysis, data ($n = 8$) were expressed as fold increase compared with untreated cells and presented as means $\pm$ SD (b). ** $P \leq 0.01$ compared with cells treated with TNF- α only.

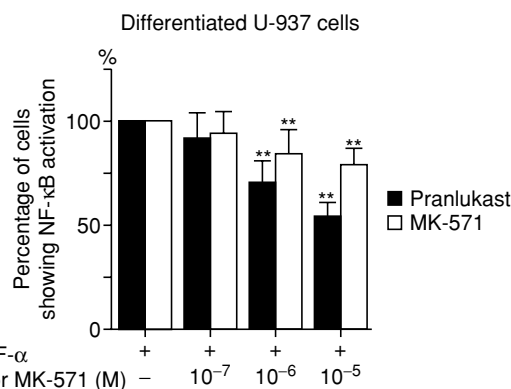


Fig. 5. Inhibitory effect of pranlukast or MK-571 on NF- κ B activation, as measured by flow cytometric analysis, in 1.3% DMSO-differentiated U-937 cells stimulated with TNF- α for 30 min, and pre-treated with pranlukast or MK-571 for 30 min before the TNF- α treatment. Data ($n = 8$) were expressed taking the value for cells treated with TNF- α only as 100% and presented as means $\pm$ SD. ** $P \leq 0.01$ compared with cells treated with TNF- α only.

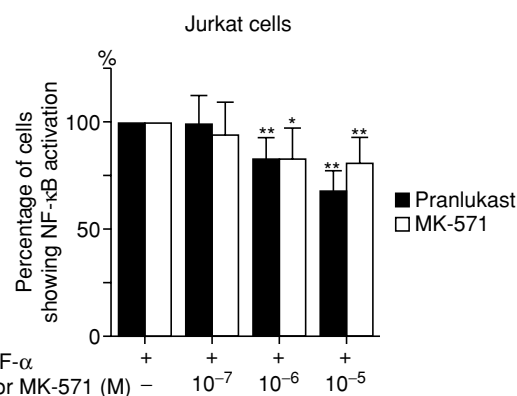


Fig. 6. Inhibitory effect of pranlukast or MK-571 on NF- κ B activation, as measured by flow cytometric analysis, in Jurkat cells stimulated with TNF- α for 30 min, and pre-treated with pranlukast or MK-571 for 30 min before the TNF- α treatment. Data ($n = 8$) were expressed taking the value for cells treated with TNF- α only as 100% and presented as means $\pm$ SD. * $P \leq 0.05$, ** $P \leq 0.01$ compared with cells treated with TNF- α only.

cells. The production of IL-6 was significantly inhibited by 10^{-5} M pranlukast and MK-571 (22.9 ± 4.0 pg/ 10^6 cells and 54.9 ± 5.0 pg/ 10^6 cells, respectively), but not by DSCG.

Discussion

Bacterial and viral infections are important triggers in a large number of children with bronchial asthma. Monocytes/macrophages are activated by bacterial products and viruses, and produce proinflammatory cytokines. Activation of monocytes/macrophages results in promotion of inflammation. Macrophages act as antigen-presenting cells that process antigens for presentation to T cells, and activated T cells produce a large variety of cytokines [18, 19]. Therefore we focused on

monocytes/macrophages and T cells as the cells responsible for infection.

Our results demonstrate that pranlukast and MK-571 modulate TNF- α -induced NF- κ B activation in 1.3% DMSO-differentiated U-937 and Jurkat cells. However, LTC₄, -D₄ and -E₄ did not activate NF- κ B in 1.3% DMSO-differentiated U-937 cells. The mechanism underlying the inhibitory effect of pranlukast and MK-571 on NF- κ B activation is unclear but it is not due to antagonism of the CysLT₁ receptor. Antagonists of the CysLT₁ receptor are a new class of drugs, which is being actively studied, and further research will expand the information on their anti-inflammatory potential. In adults administered a single oral dose of 225 mg, the C_{max} was 642.3 ± 151.0 ng/mL (1.31 ± 0.31 μ M) [20], but this value included pranlukast that is bound to plasma protein. The concentration of free pranlukast is probably at least 10-fold lower than

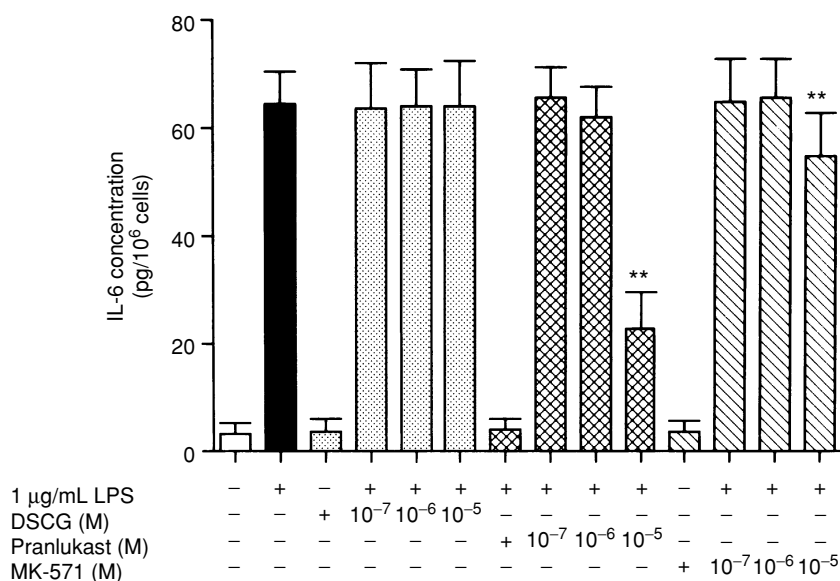


Fig. 7. Inhibitory effect of DSCG, pranlukast or MK-571 on IL-6 release, as measured by ELISA, in PBMC stimulated with LPS for 2 h, and pre-treated with DSCG, pranlukast or MK-571 for 30 min before the LPS treatment. Data ($n = 8$) were presented as means $\pm$ SD. ** $P \leq 0.01$ compared with culture fluid of cells treated with LPS only.

this [21]. Therefore the contribution of this effect to the anti-inflammatory activity of pranlukast at oral therapeutic doses in asthmatic patients remains unclear. Furthermore, the present study demonstrated that pranlukast inhibits LPS-induced IL-6 production in PBMC, and the data suggest that pranlukast suppresses proinflammatory cytokine production via inhibition of NF- κ B activation in peripheral blood monocytes and T cells. Our results suggest that pranlukast may modulate inflammation due to infection via inhibition of NF- κ B activation in monocytes/macrophages and T cells in asthmatic patients with infectious diseases.

The role of monocytes/macrophages in allergic inflammation is unclear, while T cells are important in both allergic and infectious inflammation. Further experiments, for example ones involving cells in bronchoalveolar lavage fluid, may clarify the anti-inflammatory effect of pranlukast on monocytes/macrophages and T cells in allergic inflammation. Our hypothesis could be supported by a prospective study on whether or not pranlukast decreases the frequency of attacks in asthmatic patients with associated infectious diseases.

We conclude that the inhibition of NF- κ B activation by pranlukast results in modulation of inflammation through inhibition of the production of proinflammatory cytokines by monocytes/macrophages and T cells.

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