

Montelukast Inhibits Leukotriene Stimulation of Human Dendritic Cells *in vitro*

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Key Words

Montelukast · Leukotriene · Asthma · Dendritic cell · T cell · Autologous coculture · Respiratory syncytial virus

Abstract

Background: Leukotrienes are potent inflammatory mediators which modulate immune responses and induce bronchoconstriction in susceptible individuals. Montelukast (MK) is a leukotriene receptor (CysLT1) antagonist that has been shown to prevent exacerbation of asthma. Considering the plethora of potential cellular targets for MK, specific mechanisms for its therapeutic action are still not fully understood. *In vitro*, we determined whether human dendritic cell function could be affected by leukotriene C₄ (LTC₄) treatment and whether MK had potential in modulating this response. We also studied the effect of LTC₄ in the context of response to an airway virus (respiratory syncytial virus, RSV). **Methods:** Human monocyte-derived dendritic cells (moDCs) exposed to LTC₄, MK, or both, were cocultured with autologous T cells, with or without RSV. The effects of LTC₄ and MK on cell function were determined by ELISA and proliferation assays. **Results:** Both moDCs and their precursors – monocytes – express LTC₄ receptor CysLT1, making them potential targets for MK. moDCs cultured with LTC₄ release the eosinophil chemoattractant RANTES (CCL5) and induce greater T cell proliferation. Both were blocked by the presence of MK. MK

treatment, albeit anti-inflammatory, did not interfere with the moDC-dependent T cell-proliferative responses induced by RSV. **Conclusions:** LTC₄, chronically present in the airways of asthma patients, could induce an exaggerated inflammatory response to airway infection via dendritic cell activation, which would be prevented by MK. Our study provides additional insight into the mechanisms of action of this leukotriene receptor antagonist.

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Introduction

A significant number of emergency department visits are related to infection by airway virus infections. For example, bronchiolitis, most often secondary to respiratory syncytial virus (RSV), is the most common cause of disease in infancy requiring a visit to the emergency department [1]. Exacerbations of asthma are the leading causes of emergency department visits and hospitalization in children and adults [2], and are most often due to infection with common-cold viruses [3–5]. In contrast, in the vast majority of adults and children, common colds are a simple life occurrence. In these patients, the use of anti-inflammatory medications is critical for the prevention of asthma attacks [6]. The reason why some people with asthma respond so poorly to common-cold viruses remains unclear.

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It is possible that the inflammatory response to virus infection is different in asthma patients, in whom it could lead to asthma attacks. Among the many potential immune cells, dendritic cells play a central role via the activation of CD4⁺ (helper) T lymphocytes through the presentation of antigens. CD4⁺ T cell activation includes presentation of virus antigens in an infected host within the context of the major histocompatibility complex type II (MHC-II). However, interactions via MHC-II-presented antigens are insufficient to elicit a T cell response; proper costimulatory signaling by CD80 and/or CD86 interactions with T cell surface-expressed CD28 are indispensable [7, 8]. T cell responses generated by cell-to-cell interactions are actively modulated by the cytokine/chemokine milieu present in the surrounding environment. There are many chemical signals that could potentially modulate such an immune response. Amongst such signals, leukotrienes, in particular cysteinyl leukotrienes (Cys-LTs), are some of the most potent pleiotropic inducers of inflammation [9, 10]. Cys-LTs are primarily produced and released by cells found in allergic airways, such as eosinophils, mast cells and basophils. While airway smooth muscle cells are the major target of leukotrienes, several cell types express Cys-LT receptors on their surface. Expression of Cys-LT receptors on dendritic cells is known [11], but Cys-LT modulation of their function in the context of airway virus infection has not been described. Anti-inflammatory medications like Montelukast (MK) appear to improve the response of patients to virus infection [12–14].

We have developed an in vitro model based on the generation of monocyte-derived dendritic cells (moDCs) and the isolation of cells (autologous T cells and eosinophils) from blood obtained from human donors [15, 16]. We hypothesized that leukotrienes could induce modifications of moDC function that would modulate the response of moDCs to a virus. Further, the use of the leukotriene receptor antagonist MK would prevent such modifications and a potentially exaggerated T cell response.

Material and Methods

Reagents

All leukocytes were grown in complete medium (RPMI-1640 with HEPES), supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, nonessential amino acids and antibiotic (penicillin-streptomycin-antimycotic), from Invitrogen (Carlsbad, Calif., USA). LTC₄ was purchased from Cayman Chemical (Ann Arbor, Mich., USA). Sodium MK (MK, MK-476, Singulair) was provided by Merck.

Peripheral Blood Mononuclear Cell Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human donors with no reported atopy. After red blood cell sedimentation using dextran, the leukocyte-rich supernatants were subjected to density centrifugation on Ficoll Paque Plus (GE Healthcare, Uppsala, Sweden). PBMCs were collected and used subsequently to obtain lymphocytes and dendritic cells, which are autologous in nature. All work proposed in this study was approved by the Health Research Ethics Board, according to the Helsinki Protocol, and the Biosafety Office of the University of Alberta.

T Lymphocyte Purification

Approximately 12×10^6 mononuclear cells from Ficoll-purification middle layer (PBMCs) were placed per well in 6-well plates in RPMI supplemented with 10% FBS; cells were allowed to attach to the wells for 2 h at 37°C, 5% CO₂. Unattached cells, representing a mixed lymphocyte population, were removed and run through a Nylon wool column to remove B lymphocytes. Flow-through cells were counted and stored at –80°C in FBS with 10% DMSO for later use in cocultures. Lymphocyte yield obtained this way is >90% pure CD3⁺, with a 60:40 CD4:CD8 ratio, which we have confirmed by flow cytometry, and had a viability greater than 90%. For the experiments involving coculture with moDCs, T cells were frozen in 90% FBS 10% DMSO at –80°C until required. Frozen cells had approximately 60% viability; dead cells were removed by low-speed centrifugation; cell numbers were corrected and correspond to living cells only.

moDC Preparation

We have described the protocol to generate dendritic cells from monocytes before [16]. Briefly, 6-well plates containing adherent cells, from which lymphocytes were previously removed, were washed to further remove nonadherent cells; GM-CSF (10 ng/ml) and IL-4 (12.5 ng/ml) were added to each well for the generation of moDCs. Plates were incubated for 6 days (37°C, 5% CO₂). Resulting semidetached cells were collected and irradiated to prevent proliferation. Dendritic cells obtained in this manner were >93% pure, >90% viable, and consisted in immature, CD11c⁺ HLA-DR⁺ CD80^{low} CD86^{low} dendritic cells. To resemble physiological exposure to leukotrienes found in asthma patients, for some purposes we also added 10 nM LTC₄ to monocytes after 24 h in culture. LTC₄ dose was based on observed levels of release from eosinophils in our laboratory and also from data in the literature [17, 18]. We tested 1–300 nM but found the most consistent results with 10 nM. Similarly, to try to emulate physiological conditions of users of Singulair, 10 μM MK was added 30 min before LTC₄ treatment; this dose is within the detected levels found in human serum after MK administration [19].

Expression of Leukotriene Receptor CysLT1 in T Cells and MoDCs

We sought to determine whether the CysLT1 receptor was expressed in our moDCs. Such expression would make these cells susceptible to stimulation by Cys-LT, such as LTC₄ and LTD₄, and also to the blocking effect of MK on this receptor. For this purpose, we used RT-PCR, using intron-spanning primers CysLT1F (5'-GAC AGT AAA CTG TGG ACC TTG-3') and CysLT1R (5'-ATG TCT GCT TTG TGC CTC T-3'). One microgram of RNA isolated from human dendritic cells using RNeasy kit (Qiagen)

was used for with M-MLV reverse transcriptase and oligo-dT. cDNAs were subjected to PCR for 30 cycles (95°C 30 s, 62°C 30 s, 72°C 60 s) and products were analyzed by agarose gel electrophoresis. Expected product size was 133 bp.

Cytokine and Chemokine Release Measurement of Supernatants from moDCs

To determine the activation status of moDCs and their potential release of immunologically relevant mediators, supernatants were collected from cell mono- and cocultures after LTC₄/MK treatment and exposure to RSV. Levels of IFN- α , IFN- γ , IL-12p70, IL-4, IL-13, IL-10, GM-CSF, TNF and RANTES were measured using Searchlight Protein Array Sample Testing Service (Aushon Biosystems Inc., Billerica, Mass., USA).

Cocultures of Lymphocytes and moDCs

Lymphocytes were thawed, washed with 10% FBS in RPMI, and added to 96-well plates (2×10^5 cells/well). Autologous moDCs were added to the 96-well plates (5,000/well) after washing. Plates containing various combinations of cells (lymphocytes alone, moDCs alone, or lymphocytes with moDCs) were incubated overnight. The next day, RSV (type A2, 4×10^5 PFU per well) was added to the wells. Cocultures were incubated for 7 days (37°C 5% CO₂) to allow for lymphocyte activation. Also, T cell proliferation was measured as BrdU incorporation in a 24-hour period with a BrdU Cell Proliferation ELISA chemiluminescent Kit (Roche, Mannheim, Germany), using an FLx800 luminometer (Biotek, Winooski, Vt., USA) and KC4 software; proliferation was determined as relative light units and converted into units relative to the control (T cells with untreated moDCs).

Statistical Analysis

Data were analyzed by ANOVA with Bonferroni correction (RANTES quantification) or Dunnett's posttest (proliferation assays, all groups compared to control) to identify statistical differences, using Prism version 3.0 (GraphPad Software Inc., La Jolla, Calif., USA).

Results

Dendritic Cells, but Not T Cells, Express Leukotriene Receptors

While in the literature there are reports that peripheral blood leukocytes and monocyte-derived macrophages express Cys-LT receptors [20, 21], these experiments were not conducted on purified T cells, or on moDCs as per our protocol. Expression of Cys-LT receptors would make these cells susceptible to modulation by Cys-LT such as LTC₄, and also to the blockade exerted by MK over this receptor. By RT-PCR, we found that CysLT1 receptor is expressed, at least at mRNA level, in monocytes and dendritic cells, as well as eosinophils, but not in our purified T cells (fig. 1).

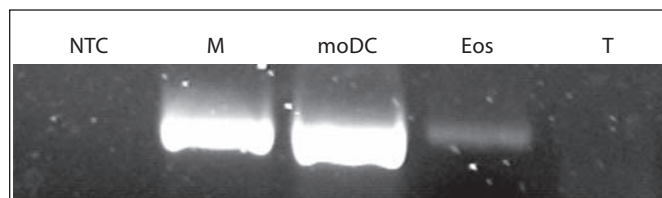


Fig. 1. Leukotriene receptor CysLT1 is expressed in human monocytes, moDCs and eosinophils but not in T lymphocytes. We obtained monocytes (M), moDCs, eosinophils (Eos) and T cells (T) from human blood. RNAs extracted from these cell types were used for reverse transcription with M-MLV reverse transcriptase and oligo-dT. cDNAs were subjected to PCR using intron-spanning primers CysLT1F and R. Product size was 133 bp as expected. CysLT1 receptor expected products were found in all analyzed cell types except for T cells and the negative template control (NTC).

Dendritic Cells Exposed to Leukotrienes Release RANTES, Which Can Be Blocked by MK

In allergic patients, it would be expected that leukotrienes would be present in tissue. Considering that dendritic cells express CysLT1 receptor, this observation suggests that dendritic cells in allergic hosts could be modified in their phenotype and function by Cys-LT stimulation during their generation and/or maturation. We measured the release of IL-1 β , IL-2, IL-8, IL-10, IL-12p70, IFN- γ , TNF and RANTES in the supernatant of moDCs generated in the presence of LTC₄, and found increased release of all of these cytokines; however, only RANTES release reached statistical significance (fig. 2). This release was not seen in dendritic cells that had been pretreated with MK before LTC₄, suggesting MK inhibited the effect of LTC₄ through CysLT1 receptor during moDC generation.

Dendritic Cells Exposed to Leukotrienes Induce T Cell Proliferation, Which Can Be Blocked by MK

We then hypothesized that LTC₄ and/or MK treatment during moDC generation could alter the capacity of these moDCs to stimulate T cells. To test this hypothesis, we measured the baseline degree of T cell proliferation in cocultures with autologous moDCs, generated in the presence of LTC₄ and/or MK. Under autologous conditions, T cell proliferation is expected to be low, for there are no MHC disparities that would trigger spontaneous proliferation as seen in allogeneic mixed cultures. LTC₄-treated moDCs consistently induced a greater degree of T cell proliferation compared to untreated moDCs (fig. 3). This induction of proliferation was not found if MK was

present in the wells before LTC₄ was added (fig. 3). We also found that baseline T cell proliferation was slightly lower when DCs had been treated with MK (fig. 3).

T Cell Proliferation in Response to RSV Is Not Impaired when Dendritic Cells Are Exposed to MK

Given the observation that MK could reduce the ability of dendritic cells to stimulate baseline T cell proliferation, we analyzed whether T cells cocultured with MK-treated dendritic cells could still respond to the presence of a respiratory virus. The degree of T cell proliferation was measured in cocultures of moDCs with or without MK treatment and RSV. Similar to our past data [16], T cells proliferate in response to RSV, but only when cocultured with autologous moDCs (fig. 4). The use of MK-generated moDCs did not alter T cell proliferation to RSV, suggesting the proliferative capacity of T cells is not impaired, nor is the antigen presentation ability of dendritic cells treated with MK (fig. 4).

Discussion

Major differences exist in the outcome of common-cold viral infections in asthma patients when compared to healthy individuals. Among the many allergic mediators in asthmatic airways, the Cys-LT family plays a prominent role [22–24]. Even in healthy individuals, the presence of mast cells in tissue would create the potential for release of Cys-LT. In allergic patients, where increased mast cell, basophil and eosinophil activity exists, there are increased levels of Cys-LT in tissue. Further, airway viral infections in allergic hosts are associated with increased Cys-LT levels [25–27]. It has been reported in the human lung that expression of the leukotriene receptor CysLT1 is almost entirely confined to the airway smooth muscle cells and tissue macrophages [21]; however, similar to previous reports [28], we found expression of the CysLT1 receptor on dendritic cells, which makes them an important target for Cys-LT and anti-Cys-LT therapy in patients suffering from allergic asthma.

In this report, we show that human moDCs can respond to stimulation by LTC₄, releasing RANTES. RANTES (CCL5) is a key chemokine for the recruitment of allergic effector cells, such as eosinophils, to inflamed tissue [29], and it has been reported to be increased in asthma exacerbation and RSV infection [30, 31]. The release of RANTES in our system was blocked by MK pretreatment. Our evidence supports previous work from Okunishi et al. [32], who found that Cys-LTs potentiate the release of

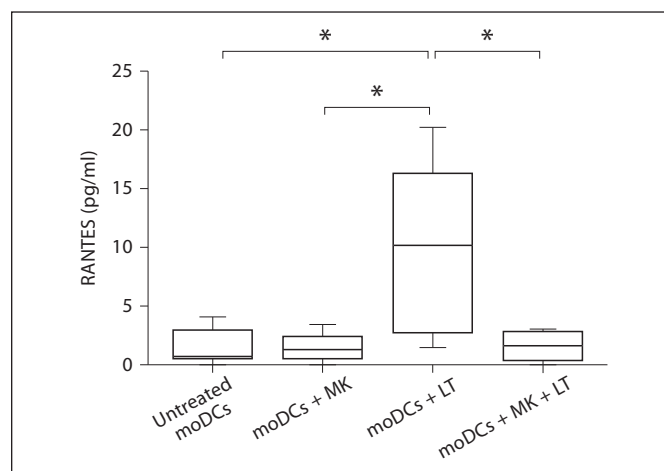


Fig. 2. RANTES release is induced in moDCs stimulated by LTC₄. Cytokine and chemokine release was measured in the supernatants of moDCs generated in the presence of LTC₄ (moDCs + LT), MK (moDCs + MK), or both (moDCs + MK + LT). MK alone did not have an effect on RANTES release; however, LTC₄ induced the release of RANTES, an effect inhibited by pretreatment with MK (n = 4 each). Untreated moDCs. Error bars represent median and interquartile range, * p < 0.05.

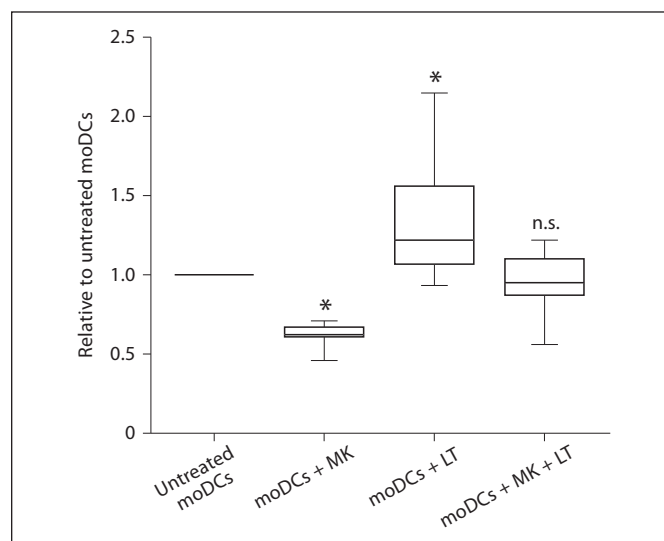


Fig. 3. LTC₄-treated moDCs (moDCs + LT) induce T cell proliferation in cocultures, which is prevented by MK. T cell proliferation was measured in cocultures of moDCs generated with MK and/or LTC₄. LTC₄-treated moDCs induced T cell proliferation compared to untreated moDCs while MK had the opposite effect (moDCs + MK). LTC₄-induced proliferation was not seen when developing moDCs were pretreated with MK (moDCs + MK + LT). Proliferation was measured using BrdU Chemiluminescent ELISA, and is expressed as relative units compared to untreated moDCs (control). Error bars represent median and interquartile range; n = 7 each, * p < 0.05, comparing each group to untreated moDCs.

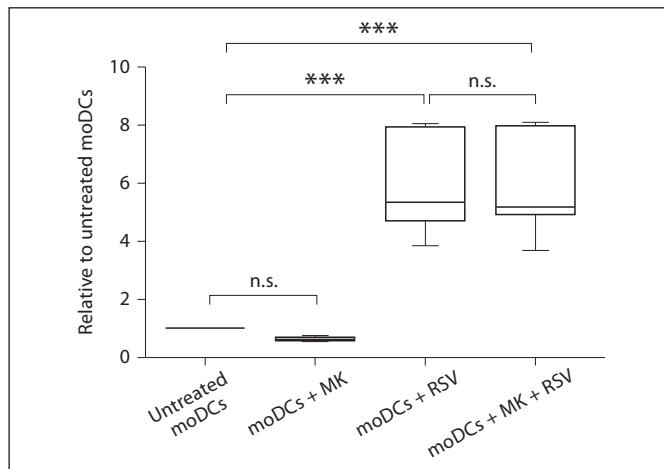


Fig. 4. MK pretreatment of moDCs does not impair response to RSV. MK pretreatment of moDCs did not affect T cell responses to RSV (moDCs + MK + RSV compared to moDCs + RSV). Proliferation was measured using BrdU Chemiluminescent ELISA, and is expressed in units relative to the control (untreated moDCs). Error bars represent median and interquartile range; $n = 7$ each, *** $p < 0.001$.

cytokines as well as the antigen presentation ability of DCs in murine lung. Our findings could also help explain the results from at least two independent groups. Kloepper et al. [33], using experimental infection of allergic-asthma patients with rhinovirus 16, found that, after infection, eosinophil numbers during convalescence were lower in individuals that were pretreated with MK. Also, in a mouse model of infection with RSV, Han et al. [34] reported a similar finding regarding the reduction in eosinophil numbers in the airways with MK treatment. However, it is also noteworthy that, in a model of experimental infection with rhinovirus 16, Kloepper et al. [33] found that, while MK did not alter viral titer and shedding, upper airway illness, cold symptom scores and virus-induced asthma symptoms, it helped prevent the drop in peak expiratory flow induced by rhinovirus 16 and it also decreased sputum eosinophilia during the recovery phase, both of which have beneficial effects on the overall potentially harmful immune response to common-cold viruses that results in asthma exacerbations.

In addition to the potential of increased recruitment of allergic effector cells, we hypothesized that Cys-LTs might alter the ability of moDCs to stimulate T cells. Our human moDCs generated in the presence of LTC₄ stimulated increased baseline T cell proliferation, which again was blocked by MK pretreatment. In addition, MK treatment

of moDCs appears to reduce stimulation of T cells. It is important to note that MK and leukotriene treatments were restricted to the monocytes while differentiating into dendritic cells, and were not present during T cell co-cultures. Further, even though moDCs have the ability to produce 5-lipoxygenase, the rate-limiting enzyme in Cys-LT synthesis [35], we detected no Cys-LT release when T cells were cocultured with moDCs (data not shown).

We have shown that T cell activation is a critical factor in the allergic response to virus infection [16, 36]. We believe that in the case of most healthy individuals, virus is cleared after a mild inflammatory process with few or no sequelae. In contrast, in allergic-asthma patients, the abnormal inflammatory response to virus results in airway hyperresponsiveness and asthma exacerbation [16]. As such, we consider the alterations to moDC function as a result of Cys-LT treatment relevant and in accordance with our hypothesis. Further, it was interesting that MK appears capable of blocking the effects of Cys-LT. However, it is also critical that this anti-inflammatory effect does not affect viral clearance. We found that MK does not impair moDC-dependent virus-induced proliferation to a respiratory virus (fig. 4), a key step in the response that leads to virus clearance. This may explain to some extent how the use of MK during the virus seasons improved outcomes [12]. We will be pursuing these hypotheses in future work.

Overall, we describe an important effect of Cys-LTs on human dendritic cell function, which is prevented by MK in vitro. These data are novel and we believe they better reflect the actual conditions of asthma patients, where dendritic cells could be generated in the allergic airway tissue from myeloid progenitors. Understanding the effects of leukotrienes on dendritic cell function and their role in common-cold infections will increase our ability to target therapy and improve outcomes in allergic-asthma patients.

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Disclosure Statement

Merck Company personnel did not participate in the design of the experiments, collection or analysis of the data, or in the preparation of the manuscript for publication. The authors have no conflicting financial interests.

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