

Biological effects of montelukast, a cysteinyl-leukotriene receptor-antagonist, on T lymphocytes

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Summary

Background Montelukast (MNT), a cysteinyl-leukotriene receptor (Cys-LTR) antagonist, has anti-inflammatory activity in the treatment of allergic diseases. If this effect is due only to blocking leukotrienes or also owing to inhibiting proliferation and survival of inflammatory cells, is actually unknown.

Objective Testing the hypothesis that MNT could influence T lymphocyte functional behaviour *in vitro*.

Methods Normal T lymphocytes were analysed for surface expression of Cys-LTR₁ and Cys-LTR₂ by means of monoclonal antibodies (mAbs), in the resting state and after activation with T helper type 2 cytokine or T cell receptor (TcR) stimulation. Proliferative activity, as well as IL-4 and IFN- γ production, were simultaneously determined in samples exposed to molar concentrations of MNT from 10^{-8} to 10^{-5} . Programmed cell death in cultured samples was evaluated by means of propidium iodide and fluorescein isothiocyanate-conjugated anti-Annexin V mAb staining. The complementary DNA microarray technique was adopted to identify gene products involved in apoptosis induction.

Results Resting T cells expressed low levels of Cys-LTR. Upon anti-CD3 mAb activation, a progressive increase in Cys-LTR₁ and -LTR₂ expression was observed. Exposure to MNT reduced proliferative response to TcR engagement, increased IFN- γ production and led to apoptosis at minimal concentrations of 10^{-6} M. A progressive loss in BAD and B cell lymphoma/leukaemia-2 activities, and an increase in the expression of CD27, TRAF3, TRAIL, p53 and *Fas* genes were also observed.

Conclusions Biological effects of MNT delineate a complex picture of gene activation and repression, probably induced by Cys-LTR blockade. The induction of apoptosis in allergen-specific T cell population, as a final result, appears fundamental in the treatment of asthma.

Keywords allergic diseases, apoptosis, cDNA, cysteinyl-leukotriene receptor antagonist, inflammation, T-cell clones

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Introduction

Biological effects of leukotrienes (LTs) require the synthesis and expression of receptors modulating their action on target cells and tissues [1]. Many inflammatory cells synthesize LT. The generation of cysteinyl LTs (Cys-LTs) LTC₄, LTD₄ and LTE₄ is amplified by many substances implicated in the pathogenesis of inflammatory diseases, such as IL-4, IL-5, IFN- γ , granulocyte-macrophage colony-stimulating factor (GM-CSF), eotaxin, endothelin and transforming growth factor (TGF)- β [2]. Two subtypes of Cys-LT receptors (Cys-LTR₁ and Cys-LTR₂) have been cloned and found at the surface of many inflammatory cell types, including T lymphocytes [3, 4]. The Cys-LTR₁ is most highly expressed

in the spleen, peripheral blood leucocytes, interstitial lung macrophages, and airway smooth muscle, whereas Cys-LTR₂ is found in the heart, adrenal, placenta, spleen, peripheral blood leucocytes and less strongly in the brain. Cys-LTRs are differently involved in transendothelial migration of CD34⁺ haematopoietic progenitor cells, arterial smooth muscle contraction and many other functional activities (recently reviewed in Ref. [5]). They also differ with respect to the sensitivity to classical pharmacological antagonists, which is greater for Cys-LTR₁ [5].

There is little experimental evidence that Cys-LT are involved in the generation of inflammatory infiltrate in the airways of asthmatic subjects. However, it has been shown that: (i) Cys-LT may be synthesized and released during T cell activation through the T cell receptor (TcR)/CD3 complex, following translocation of 5-lipoxygenase to the cell membrane [6]; (ii) inhalation of Cys-LT increases eosinophil number in the respiratory epithelium and in the sputum of

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asthmatic subjects [7, 8]; and (iii) treatment with anti-inflammatory agents, including Cys-LTR antagonists [9], corticosteroids [10] and theophylline [11], reduces the percentage of T lymphocytes and eosinophils in the bronchoalveolar lavage (BAL) in asthmatic patients.

The relevance of T lymphocytes in initiating and driving the allergic immune response is widely accepted [12], but the molecular basis for their persistence at the sites of inflammation has been only partially clarified [13]. One important mechanism underlying lymphocyte control is programmed cell death (apoptosis), which occurs in every immune response [14]. T cells have at least two apoptotic pathways: active death, which is antigen driven, and passive death, which may be because of lymphokine withdrawal or other mechanisms [15]. Cross-linking of *Fas*/APO-1 (CD95), a 45 kDa protein belonging to the TNF receptor family, with its ligand (*FasL*) induces apoptosis in lymphocytes [16] and provides a mechanism for removal of antigen-activated T cells [17, 18], thus facilitating resolution of the inflammatory response [19].

Allergen-specific T lymphocytes isolated from the respiratory tract of asthmatic patients display a defective *Fas* surface expression, which is secondary to suppression of *Fas* mRNA [20], and then persist at the site of allergic inflammation. T-helper type 2 (Th2)-type cytokines are primarily involved in *in vitro* induction of *Fas* mRNA suppression in a dose-dependent manner, which resembles what usually occurs in allergic inflammation *in vivo* [21]. Among 5-lipoxygenase products, 5-HETE [22] and LTB₄ [23] display an anti-apoptotic activity on several cell types including leucocytes, a property that is also common to Th2-type cytokines [24]. Thus, many cellular and soluble mediator loops seem to act synergistically in prolonging the survival of inflammatory cells.

The putative anti-inflammatory mechanism of action of Cys-LTR antagonists is still poorly understood. The present work investigated the *in vitro* expression of Cys-LTR at the surface of T lymphocytes and determined the effects of montelukast (MNT), a Cys-LTR₁ antagonist, on their proliferative response and/or apoptotic death.

Methods

Cell sources and culture reagents

For *in vitro* experiments, a series of 15 healthy blood donors was recruited among those referring to our Blood Bank, who had previously given their written informed consent. All experiments were performed in triplicate. Normal T lymphocytes were negatively selected from peripheral blood leucocytes by monocytes adhesion followed by sorting of cells expressing CD14, CD16, CD19 and CD161 antigens by means of magnetic bead-coated monoclonal antibodies (mAbs) (Serotec, Kidlington, Oxford, UK), adsorbed over a Vario Macs (Miltenyi Biotec, Bergisch Gladbach, Germany) column. Negatively selected highly purified T cells always consisted of up to 94% CD3⁺ lymphocytes. In addition, a Jurkat T cell line (LGC Promochem Limited, Teddington, UK), and a series of allergen-specific T cell clones ($n = 5$, all CD4⁺) derived from the BAL fluid of asthmatic patients who were sensitive to *Dermatophagoides pteronyssinus* were used.

All cultures were performed in serum-free AIM-V medium (Gibco, Grand Island, NY, USA). Th2-type cytokines IL-4 (Serotec, 400 IU/mL) or IL-13 (Serotec, 5 µg/mL) were added, when required, to culture samples, alone or in conjunction with plastic-coated anti-CD3 mAb (Ortho, Raritan, NJ, USA; from 0.5 to 1 µg/mL). Peripheral blood T cell cultures, pre-activated by 24 h exposure to anti-CD3 mAb, were mixed with MNT (dissolved in absolute ethanol and adjusted to molar concentrations ranging from 10^{-8} to 10^{-5}) and incubated for an additional 72 h at 37 °C, 5% CO₂. Constitutively activated Jurkat T cell line or allergen-specific CD4⁺ T cell clones were cultured in medium alone or in medium supplemented with MNT.

Surface cysteinyl leukotriene receptors

Stable induction of surface Cys-LTR was evaluated on resting, Th2-type cytokine- or anti-CD3 mAb-activated T cells, by means of specific mAbs directed against Cys-LTR₁ and -LTR₂ (Cayman Chemical, Ann Arbor, MI, USA). Cell samples (1×10^6) were incubated on ice for 30 min with mAbs, washed three times in phosphate-buffered saline without Ca²⁺ and Mg²⁺ (Gibco) and then incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Southern Biotechnology, Birmingham, AL, USA). Fluorescence was then read on a FACScan cytofluorimeter (Becton-Dickinson, Franklin Lakes, NJ, USA) equipped with CellQuest software.

Proliferative response

Highly purified circulating T cells (0.2×10^6 cells/well) were pre-stimulated with plastic-coated anti-CD3 mAb (from 0.5 to 1 µg/mL) on 96-well culture plates (NunClone, Roskilde, Denmark) for 24 h at 37 °C, 5% CO₂. MNT was then added to some cultures at doses ranging from 10^{-8} to 10^{-5} M. The proliferative response was then evaluated by measuring ³H thymidine incorporation after another 72 h incubation. Before the end of culture time, T cells were pulsed for 16 h with 0.5 µCi H-thymidine (Amersham Biosciences, Little Chalfont, UK), and radionuclide uptake was measured by scintillation counting. Values are expressed as mean c.p.m. incorporation $\pm$ SD calculated on ten consecutive experiments performed in triplicate.

Cytokine assay

5×10^5 normal T cells were cultured as above on 96-well culture plates and exposed to MNT in doses ranging from 10^{-8} to 10^{-5} M. Supernatants were harvested after 5 days of culture. Cytokine release was determined for IL-4 and IFN- γ by commercial ELISA kits (Quantikine, R&D Systems, Minneapolis, MN, USA). Results are expressed as pg/mL $\pm$ SEM.

Apoptosis assay

Samples of enriched T lymphocytes, allergen-specific pulmonary T cell clones and the Jurkat T cell line were washed and resuspended in RPMI-1640 (Gibco) supplemented with 10% foetal calf serum, 2 mmol L-glutamine/L, 10 mmol HEPES/L, 50 U penicillin and 50 µg streptomycin/L at 10^6

cells/mL. All samples were incubated with medium alone or with plastic-coated anti-CD3 mAb (from 0.5 to 1 µg/mL) for 24 h. After washing, cell samples were exposed for a further 48 h to different concentrations of MNT (ranging from 10^{-8} to 10^{-5} M). Unstimulated and anti-CD3 mAb stimulated, with or without incubation with MNT, samples were quantitatively assessed for programmed cell death by cytofluorimetry with a fluorochrome solution containing propidium iodide or by indirect staining with mAb to human annexin V (Serotec), developed with FITC-labelled rabbit anti-mouse IgG (Southern Biotechnology), as described previously [21]. Pulmonary allergen-specific CD4⁺ T cell clones and Jurkat T cell line were cultured in medium alone or with MNT without pre-activation.

Complementary DNA microarray

To avoid interference caused by the particular functional state of T cell clones or the Jurkat T cell line, we adopted freshly isolated T lymphocytes, pre-activated with plastic-coated anti-CD3 mAb, with or without adding different MNT concentrations. The non-radioactive GEArrayTM Q series complementary DNA (cDNA) expression array filters [for apoptosis and common cytokines: HS-002N-4, HS-003N-4 (SuperArray Bioscience Corp., Frederick, MD, USA)] were used. Hybridization procedures were performed according to the manufacturer's instructions. Total RNA from our culture samples was extracted by TRIzol[®] (Invitrogen, Life Technologies, Carlsbad, CA, USA). The biotin dUTP-labelled cDNA probes were specifically generated in the presence of gene-specific primers using total RNA (4 µg/filter) and 200 U Moloney Monkey Leukemia Virus reverse transcriptase (Promega, Madison, WI, USA). The array filters were hybridized with biotin-labelled probes at

60 °C for 17 h. The filters were then washed twice with 2 × saline-sodium citrate (SSC)/1% SDS and then twice with 0.1% SSC/1% SDS at 60 °C for 15 min each. Chemiluminescent detection steps were performed by subsequent incubation of the filters with alkaline phosphatase and CDP-star substrate, as recommended by the manufacturer. All cDNA microarray experiments were performed twice with new filters and RNA was isolated at different times. The positive and negative spots were identified and matched by at least two investigators. Only the matched positive and negative results of two experiments are presented.

Statistical analysis

Due to the non-normal distribution of samples, a non-parametric one-way ANOVA test was adopted to calculate the differences in Cys-LT receptors distribution among the various T cell populations, as well as the differences in proliferative activity and cytokine secretion pattern between MNT-treated and non-treated samples. Differences in the mean percentage and SD were calculated by the Mann-Whitney *U*-test. Values of *P* below 0.05 were considered to be significant.

Results

Modulation of cysteinyl leukotriene receptors

Resting T lymphocytes displayed surface Cys-LTR₁ and -LTR₂ at percentages ranging from 0.5% to 2.5%. Incubation with medium alone did not affect Cys-LTR expression, whereas culturing with a mixture of IL-4 and -13 slightly increased the percentage of Cys-LTR positive T cells. A

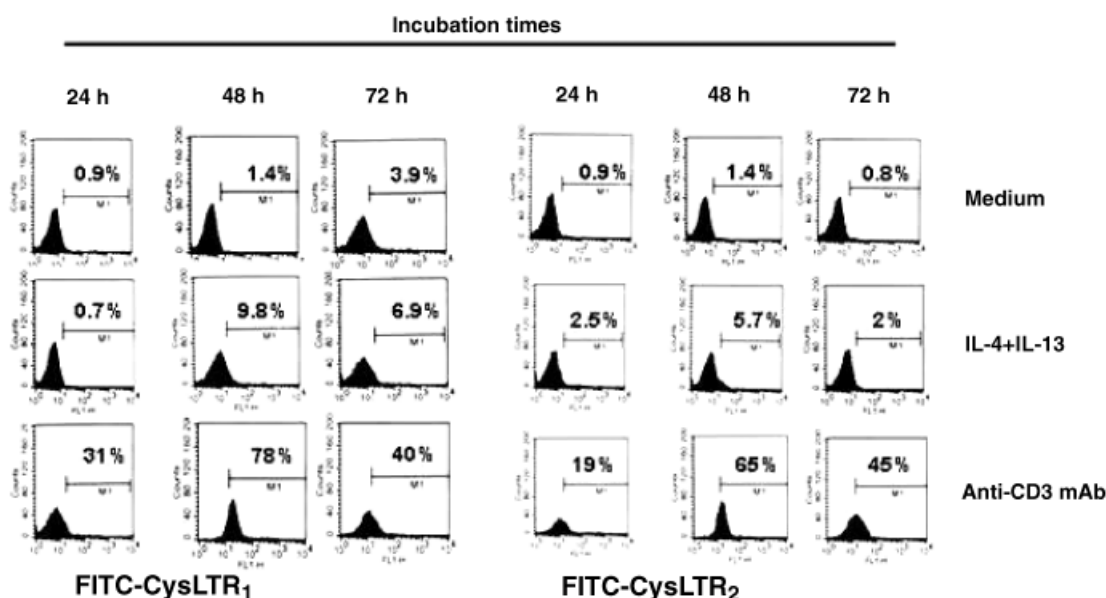


Fig. 1. Induction of cysteinyl-leukotriene receptor (Cys-LTR)₁ and Cys-LTR₂ on T cells. Cell samples (3×10^6 /mL) were evaluated for the expression of Cys-LTR by an indirect immunofluorescence technique. Time-course experiments were set up with medium alone (upper panels) in the presence of T-helper 2-type cytokines (400 U/mL IL-4, plus 5 µg/mL IL-13) added at the starting of culture (middle panels), or following T cell receptor stimulation by plastic-coated anti-CD3 monoclonal antibody (mAb) (0.5 µg/mL, lower panels). At each time-point (24, 48 and 72 h), one-third of cultured cells was harvested and stained for Cys-LTR expression. Appropriate negative controls were represented by T cells cultured in medium alone. Depicted results are obtained from a single representative experiment of those performed on all healthy subjects. Line markers were set after subtracting background fluorescence measured in samples stained with the fluorescein isothiocyanate-labelled second antibody alone.

striking effect was obtained after triggering the TcR by means of plastic-coated anti-CD3 mAb (Fig. 1). Co-administration of Th2-type cytokines at the start of culture did not significantly affect receptor expression (data not shown). Stimulation with soluble anti-CD3 mAb induced a slight, but

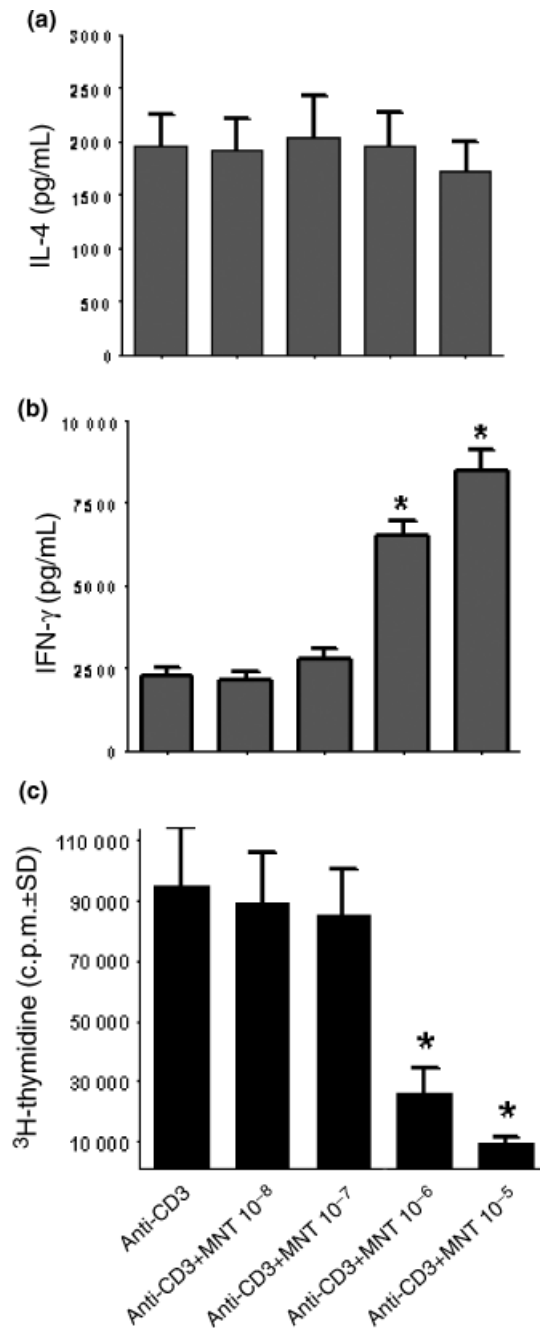


Fig. 2. *In vitro* cytokine production and T cell proliferative response to increasing montelukast (MNT) concentrations. Enriched T cell samples were cultured for 5 days with plastic-coated anti-CD3 monoclonal antibody (mAb) in the presence or not of serial dilutions of MNT, which was added 24 h after starting culture. Cytokine measurements in harvested supernatants of cell cultures demonstrated no significant changes in IL-4 (a), but a significant increase in IFN-γ production (b) at an MNT concentration of 10⁻⁶ and 10⁻⁵ M (**P* < 0.001). (c) Dose-dependent inhibition of ³H-thymidine incorporation at a starting dose of 10⁻⁶ M (**P* < 0.001) are shown. Depicted values of *in vitro* cytokine production and proliferative responses represent means ± SD obtained by subtracting the negative controls represented by T cells cultured with medium alone.

progressive, rise in the percentages of Cys-LTR-positive T cells, which was maximal after 72 h incubation (range: from 35% to 49%). Surface Cys-LTR₁ and Cys-LTR₂ were constitutively present on pulmonary allergen-specific CD4⁺ T cell clones obtained from Der p 1-sensitive subjects, in a percentage ranging from 22% to 35%. Proliferating Jurkat T cell line displayed a percentage of Cys-LTR-positive cells always greater than 60%.

Montelukast-induced functional changes in T lymphocytes

Culturing anti-CD3 mAb activated T cells with different amounts of MNT left IL-4 production substantially unchanged. IFN-γ production rose, as MNT molar concentrations were increased (Figs 2a and b). A concomitant, dose-dependent loss of T cell proliferative activity occurred, as demonstrated by the fall in ³H-thymidine incorporation after 72 h culture (*P* < 0.001 for MNT concentration starting at 10⁻⁶ M) (Fig. 2c).

Montelukast-induced T cell apoptosis

The percentage of apoptosis increased in a dose-dependent manner after exposure of anti-CD3 mAb activated normal T lymphocytes to MNT, reaching significance vs. unstimulated or anti-CD3 mAb activated cells with a 10⁻⁶ M MNT concentration (*P* < 0.001). To confirm the direct drug activity on non-*in vitro*-activated cell sources, pulmonary allergen-specific CD4⁺ T cell clones or Jurkat T cell line were cultured with the drug alone. An increase in percentage of apoptotic cells starting at 10⁻⁶ M MNT concentration was detected (Fig. 3a). Propidium iodide staining and FITC-Annexin V labelling technique gave always comparable results in evaluating the percentages of cell death in samples tested (Figs 3b and c). The drop in cell viability, which is responsible for the failure of the T cell proliferative response, correlated with the presence of apoptotic cells.

Complementary DNA microarray

The assays were performed on parallel samples pre-activated with anti-CD3 mAb. Since this technique is qualitative, attention was focused on the absolute number of T cells placed into culture wells, the time-course of drug exposure, and the absolute amounts of RNA that were finally extracted.

Compared with the negative controls (T cells exposed to anti-CD3 mAb alone), a complex picture of gene expression was evident in MNT-treated samples. Progressive loss in BAD and B cell lymphoma/leukaemia-2 (Bcl-2) activity, and a concomitant increase in the expression of CD27, TRAF3, TRAIL, p53 tumour-suppressor gene and *Fas* were found (Fig. 4a). Some other cytokine-related genes, such as fibroblast growth factor-11, insulin-like growth factor-1, IFN-γ and CD30L, were overexpressed in MNT-treated cultures (Fig. 4b). Changes were more evident at the MNT concentration of 10⁻⁵ M.

Discussion

Anti-LT agents have not been included in the treatment of allergic diseases without some concern [25]. Doubts about

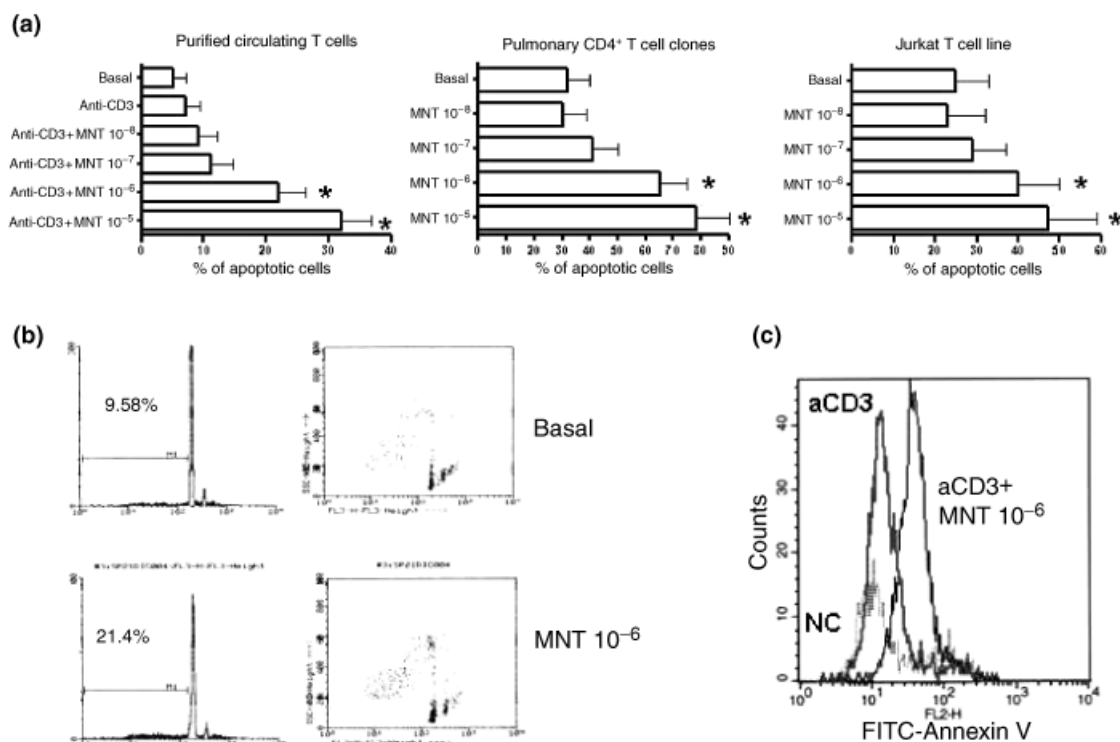


Fig. 3. Induction of T cell apoptosis by montelukast (MNT). The occurrence of apoptotic death was measured in three different settings and is depicted in (a). Purified circulating T cells were pre-activated by 24 h culturing with anti-CD3 monoclonal antibody (mAb), and then exposed to various concentrations of MNT or medium alone for a further 48 h. Pulmonary allergen-specific T cell clones and Jurkat T cell line were directly exposed to MNT at the starting of culture. Controls were represented by samples cultured in medium alone. The occurrence of programmed cell death was evident at MNT doses of 10^{-6} M or higher (* $P < 0.005$). Two representative examples of cell death measurement, as it appears with propidium iodide (b) or fluorescein isothiocyanate-conjugated anti-annexin V mAb staining (c), are shown.

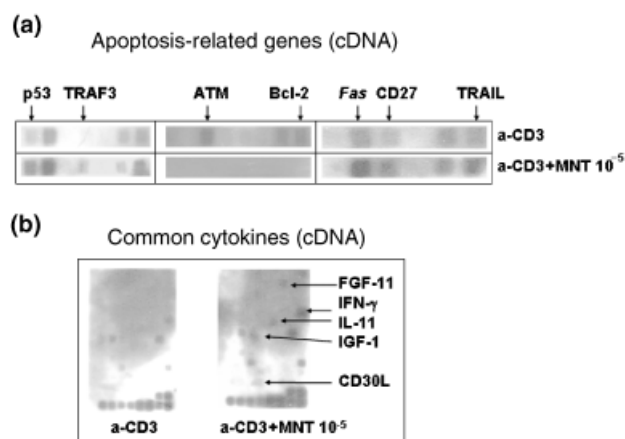


Fig. 4. Complementary DNA microarrays. Activation of genes involved in the apoptotic process was sought in anti-CD3 monoclonal antibody (mAb) activated T cells cultured in medium alone, compared with those co-exposed to montelukast (MNT) (at concentrations of 10^{-6} and 10^{-5} M, which induce apoptosis). (a) Apoptosis-related genes that display significant variations after MNT exposure are indicated by arrows. In the same samples (b) changes observed in common cytokine genes are indicated with arrows. One representative experiment of those performed on T cell samples obtained from normal blood donors is illustrated.

anti-inflammatory actions, therapeutic potency and heterogeneity of efficacy have been raised, despite a complementary [26] and hence steroid-sparing effect of Cys-LTR antagonists [27]. In the present study, a specific anti-inflammatory mechanism of action directed towards proliferative and

survival behaviour of allergen-specific T lymphocytes has been observed with MNT.

Induction of Cys-LTR at the surface of activated T lymphocytes is the pre-requisite for the action of MNT *in vitro*. Such receptors have already been found on T lymphocytes, even though percentages never exceed 10% [28]. Our data show that Cys-LTR positive T cells were increased *in vitro* by triggering the TcR, thus suggesting that only activated T lymphocytes are the MNT target. Cys-LTR induction is stable over time and results in a logarithmic enhancement of fluorescence intensity in positive cells, with plastic-coated anti-CD3 mAb being more effective than soluble anti-CD3 mAb. Interaction of T lymphocytes with antigen and antigen-presenting cell enables activated T cells to express functional Cys-LTR [12]. This finding, together with the demonstration that Th2-type cytokines partially enhance the Cys-LTR surface expression on monocytes and macrophages [29], suggests that better effects may be obtained by MNT when it is administered during antigen exposure.

The impairment in T cell proliferative responses and their partial loss of viability are the main effects of the *in vitro* interaction of MNT with its receptors. This phenomenon is dose-dependent and may involve inhibition of survival-related genes, such as Bcl-2, with the consequent induction of apoptosis in antigen-activated T lymphocytes. MNT-induced apoptosis has been demonstrated in several other cellular populations, such as neutrophils [30] and eosinophils [31]. Interfering with leukotriene synthesis or their receptors has been hypothesized to interrupt the paracrine survival loop

on cultured granulocytes from normal and asthmatic subjects [32]. Our data support the view that MNT-induced lymphocyte death does not depend on growth factors or LT withdrawal. Otherwise, MNT engagement of Cys-LTR could injure DNA, with damage resulting in Bcl-2 suppression together with partial up-regulation of p53 gene.

The concomitant inhibition of BAD expression, a pro-apoptotic member of the Bcl-2 family [33], seems to contrast with our observations. However, it should be noted that the apoptotic cells in our experimental model reached percentages of about 30–40% of the total cultured cells. In this setting, RNA extraction is performed on cultured samples that contain a significant percentage of viable cells (>50%). Since a qualitative assay was adopted in our study, down-modulation of some anti-apoptotic genes should be interpreted with caution. Living cells could have retained functional Bcl-2, whereas apoptotic cells, while retaining BAD, did not.

The up-regulation of some TNF-related genes, such as TRAF3, CD27 and TRAIL together with the maintenance of high *Fas* activity, can be interpreted as MNT-related effects. Members of the TNF protein family, such as *Fas*, are involved in the maintenance of Th2-type CD4⁺ lymphocytes at the sites of allergic inflammation [20, 21]. This mechanism does not entirely depend upon the nature of the antigenic stimulus, but is probably related to the Th2-type cytokine milieu. Indeed, IL-4 and -13 both suppress *Fas* mRNA on T cells *in vitro* [21]. Asthmatic subjects do not display *Fas*⁺ T lymphocytes in inflamed lungs, whereas *in vitro* IFN- γ stimulation up-regulates *Fas* mRNA [20, 21]. Persistent *Fas* gene activation, together with overexpression of IFN- γ and other TNF-related genes (TRAF3 [34], CD27 [35], TRAIL [36], CD30L [37]) observed after MNT exposure, could account for apoptosis induction in our experimental system. Finally, exposure of anti-CD3 mAb activated T cells to MNT produced a slight but consistent up-regulation of p53 gene. This gene inhibits cell growth through activation of the cell-cycle arrest and apoptosis [38], and probably acts by activating nuclear factor- κ B, since its loss or inhibition can abrogate p53-induced apoptosis [39].

Our results delineate a complex picture of gene activation and repression, probably induced by Cys-LTR blockade, which leads to the apoptosis of activated T cells. Inducing apoptosis as a therapeutic anti-inflammatory goal to reduce excessive cell population density or activity appears to be in line with endogenous processes for resolving inflammation [40]. The engulfment of apoptotic cells by macrophages and other non-professional phagocytic cells before necrosis prevents the release of dangerous inflammatory signals, thus facilitating repair of damaged tissues.

In vivo studies with MNT have produced promising results on the cellular composition of induced sputum [41, 42, 43], but the protective effect against methacholine or histamine-induced airway hyper-responsiveness is less amazing [44]. Such discrepancies may reflect the lack of correlation among various biomarkers of asthma. Alternatively, the current dosing of MNT may be inadequate for patients with severe chronic asthma or acute exacerbations [45]. As higher levels of Cys-LT and their receptors are expected under such conditions, it would seem reasonable, as our *in vitro* study suggests, to re-examine dosing of this drug.

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