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LTD₄ and TGF- β ₁ Induce the Expression of Metalloproteinase-1 in Chronic Rhinosinusitis via a Cysteinyl Leukotriene Receptor 1-Related Mechanism

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Abstract: *Background:* Cysteinyl leukotrienes (CysLTs) play a crucial role in the pathogenesis of airway remodeling. The use of CysLTs receptor antagonists has been included in the management of asthma and rhinitis. However, despite the action of these compounds on leukotriene production has been well documented, their role in airway remodeling remains unclear. *Objective:* We aimed to investigate the capability of the leukotriene receptor antagonist Montelukast to inhibit MMPs release after CysLTs stimulation in nasal tissue fibroblasts. *Methods:* Fibroblasts were isolated from sinunasal tissue collected from five patients suffering of chronic rhinosinusitis without nasal polyposis. Cells were cultured and stimulated first with LTC₄ and LTD₄ (10^{−10}, 10^{−8}, 10^{−6} M) using as pre-stimulus 10 ng/mL of: IL-4, IL-13, or TGF- β ₁ and in presence or absence of Montelukast (10^{−10}, 10^{−8}, 10^{−6} M). To evaluate the regulation of MMP-1 and TIMP-1 we used enzyme immunoassays and to evaluate CysLT₁ receptor we used real time PCR. *Results:* LTD₄ but not LTC₄ induced production of mRNA for CysLT₁ receptor in a dose dependent manner and with an additive effect when the cells were primed with TGF- β ₁. TNF- α , IL-4, and IL-13 did not influence the expression of the receptor. Levels of MMP-1 but not of TIMP-1 were statistically enhanced in cells primed with TGF- β ₁ and stimulated with LTD₄. Montelukast significantly decreased Cys-LT₁ receptor and MMP-1 concentrations in a dose-dependent way in cells stimulated with LTD₄ and TGF- β ₁ separately and when they were applied together. *Conclusion:* The leukotriene pathway may play an important role in extra-cellular matrix formation in an inflamed environment, such as chronic sinusitis and, consequently, leukotriene receptor antagonists such as Montelukast may be of great benefit in management of this disease.

Keywords: chronic rhinosinusitis; cysteinyl leukotriene receptor 1; fibroblasts; metalloproteinase 1; Montelukast

1. Introduction

Chronic rhinosinusitis (CRS) is a heterogeneous group of diseases characterized by chronic inflammation of the nasal and sinunasal mucosa, persisting longer than 12 weeks. Recent studies have demonstrated that the spectrum of the disease can be differentiated into distinct subgroups based on clinical parameters and on the characterization of the inflammatory response [1]. Although chronic

rhinosinusitis without nasal polyps (CRSsNP) shows mainly a neutrophilic, T_H1 -driven inflammatory profile, chronic rhinosinusitis with nasal polyps (CRSwNP) is mainly characterized by a T_H2 -skewed, eosinophilic inflammation [1]. However, in both CRS phenotypes, the turnover of the extracellular matrix (ECM) is disturbed by the release of inflammatory mediators such as TGF- β_1 , MMP-7, MMP-9, and TIMP-1 in nasal secretions. Resulting in an abnormal tissue remodeling characterized by mucosal thickening, fibrosis, and/or edema [2,3].

ECM remodeling processes consist of a complex interaction between various cell types and a large number of enzymes including tissue serine proteases and the family of matrix metalloproteinases (MMPs) [4]. Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent endopeptidases which can collectively degrade almost all ECM components [5]. They are produced by structural (fibroblasts, endothelial, and epithelial cells) and inflammatory cells (eosinophils, macrophages, neutrophils, and lymphocytes) and the gene expression is tightly regulated by cytokines and growth factors.

The role of MMPs in physiology has been implicated in both normal and pathological structural processes, such as embryogenesis, cell migration, tissue repair, and tumor necrosis [6,7]. The increased expression of MMPs in nasal polyps, COPD, and asthma and the correlation with pathological changes of the airways, together with their action in wound repair have been the basis to suggest the possible role of these compounds in microvascular permeability, cell transmigration, and ECM airway remodeling [8]. Although this evidence exists, the *in vivo* function of these MMPs in airway diseases remains partially unclear.

Cysteinyl leukotrienes (CysLTs) are lipid mediators produced after the release of arachidonic acid from cell membrane phospholipids which is then modified by the 5-lipoxygenase (ALOX5) enzyme to yield LTB₄ or to the cysteinyl-leukotrienes (CysLTs) LTC₄, LTD₄, and LTE₄ [9]. These compounds are released by cells involved in the inflammatory response (mast cells, basophils, eosinophils, neutrophils, and macrophages) and constitute important pro-inflammatory mediators in asthma and other chronic inflammatory diseases [9,10]. Their action is mediated by the binding to CysLTs receptors (CysLT1 and CysLT2 receptors), which are expressed not only in inflammatory and immune cells, but also in structural cells. Binding of CysLTs on their receptors on structural cells results in tissue edema, mucus secretion, bronchoconstriction, and severe impairment in tissue remodeling [11].

In nasal tissue of CRSsNP patients, levels of the CysLTs, the enzymes involved in their synthesis (ALOX5 and LTC₄S), and both CysLT1 and CysLT2 receptors are significantly upregulated compared to controls [12,13]. Several *in vitro* experiments have suggested that CysLTs may induce airway smooth muscle proliferation [14,15], alter fibroblast function [16,17] and induce extracellular matrix [18,19].

Taking into account the important role of leukotrienes in airway inflammation, and especially in asthma, several anti-LTs modifiers have been evaluated in the management of this pathology [20–22]. Leukotriene modifiers include three different groups of drugs: (i) specific inhibitors of FLAP; (ii) inhibitors of ALOX5; and (iii) CysLT1 receptor inhibitors. However, the role of these compounds in upper airway remodeling remains unclear. In this study we evaluated the capability of exogenous CysLTs (LTC₄ and LTD₄) to influence the expression of MMPs in nasal tissue fibroblasts isolated from patients with chronic rhinosinusitis without nasal polyposis (CRSsNP) and evaluate if the leukotriene receptor antagonist Montelukast may modify this effect and, consequently, may have a potential role in regulation upper airway tissue remodeling.

2. Materials and Methods

2.1. Patients

Fibroblasts were isolated from nasal mucosa tissue obtained from CRSsNP patients ($n = 5$) who were scheduled for functional endoscopic sinus surgery at the Department of Otorhinolaryngology at Ghent University Hospital. The diagnosis of CRSsNP was based on history, clinical examination, nasal endoscopy, and computed tomography (CT-Scan) of the paranasal cavities according to the EPOS

guidelines [23]. None of the patients had asthma or allergies as assessed by the guidelines (GINA) for the diagnosis and management of asthma [24] and results of a skin prick test. The study was approved by the ethical committee of Ghent University Hospital and all patients gave informed consent before their participation.

2.2. Reagents

Dulbecco's PBS, penicillin-streptomycin (penicillin, 5000 IU/mL; streptomycin, 50 µg/mL), and trypan blue (0.4% solution in PBS) were obtained from Invitrogen (Paisley, UK). Minimum essential medium (MEM), Opti-MEM I reduced serum medium, L-glutamine (200 mM), trypsin-EDTA (1X), and fetal bovine serum (FBS) (qualified; origin, Thermo Fisher Scientific, Waltham, MA, USA) were purchased from Gibco (Life Technologies, Carlsbad, CA, USA). Ultrosor™ G serum substitute (5%) was obtained from BioSeptra (Port Washington, New York, NY, USA). Interleukin-4 (IL-4) and interleukin-13 (IL-13) were obtained from R and D Systems (Minneapolis, MN, USA). SYBR Green I qPCR Master mix, Aurum Total RNA, and Script cDNA synthesis kits were obtained from Bio-Rad Laboratories (Berkeley, CA, USA). Cysteinyl leukotrienes C₄, -D₄ (LTC₄, LTD₄) and the cysteinyl leukotriene receptor 1 (CysLT1) inhibitor Montelukast were purchased from Cayman Chemicals (Ann Arbor, MI, USA).

2.3. Isolation of Nasal Mucosa Fibroblasts

Nasal sinus tissues, obtained from patients with chronic rhinosinusitis, during surgical operations were rinsed several times with Opti-MEM I supplemented with 5% FBS, 5% Ultrosor G, 2 mmol/L glutamine, and penicillin (50 IU/mL)–streptomycin (50 µg/mL), and cut into small pieces (approximately 1 mm²). Diced specimens were then plated (density, 9 pieces/6-well tissue culture dish) and incubated in a humidified atmosphere containing 5% CO₂ at 37 °C, until a monolayer of fibroblast-like cells was observed to be confluent. Then, the explanted tissues were removed, and cells were trypsinized and re-plated into 250-cm² tissue culture Falcon tubes at a final volume of 5 mL. The culture medium was then changed every three days for 2–3 weeks until 90% confluence was obtained. Subsequently, the cells were split and stained with antibodies against vimentine, cytokeratin, and α-smooth muscle actin to exclude contamination with epithelial cells.

2.4. Cell Stimulation

Cells (1×10^6) were cultured with 10 ng/mL of IL-4, IL-13, TGF-β₁, and TNF-α alone or in combination with (10^{-6} , 10^{-8} and 10^{-10} M) of LTC₄ or LTD₄ independently during 24 h at 37 °C in 5% CO₂. For inhibition experiments, sinunasal fibroblasts were first cultured in presence of Montelukast (10^{-8} , 10^{-10} , 10^{-11} M) for 60 min, and after, stimulated with 10 ng/mL of TGF-β₁ or in combination with LTD₄ at 10^{-6} M) for 24 h, at 37 °C in 5% CO₂. After all stimulations, supernatants were collected and stored at −20 °C until use. Cells were resuspended in lysis buffer for posterior RNA extraction, and stored at −80 °C.

2.5. Measurement of MMP-1, TIMP-1, and Cysteinyl Leukotrienes

Protein levels of matrix metalloproteinase-1, -2, -3, -7, and -9 (MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9) and TIMP-1 were quantified by the human total MMPs and TIMP-1 Quantikine ELISA kits (R and D Systems) following manufacturer's instructions.

2.6. Real-Time qPCR for CYSLT1 Receptor

Cell pellet was homogenized in Tri-reagent buffer (Sigma-Aldrich, MO, USA), 1 mL per 50–100 mg of tissue and total RNA was isolated using the Aurum total RNA Kit (BioRad Inc. Laboratories) following the manufacturer's instructions. cDNA was synthesized from 2 µg of total RNA using the iScript cDNA synthesis kit Bio-Rad Inc. Laboratories). Amplification reactions were performed on an

iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories) using a primer set for human cysteinyl leukotriene receptor 1 (Table 1). PCR reactions contained 20 ng of cDNA (total RNA equivalent) of unknown samples, 1X SYBR Green I Master mix (Bio-Rad Laboratories) and 250 nM of primer pairs in a final volume of 20 µL. PCR protocol consisted of one cycle at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The expression of two housekeeping genes, Beta actin (ACTB) and hydroxymethyl-bilane synthase (HMBS), were used to normalize for transcription and amplification variations among samples. Primer sequences and qPCR conditions are reported previously [25]. Relative normalized quantities were calculated from the obtained C_q values using the qBase software [26].

Table 1. Primer sequence for quantitative real-time PCR.

Gene	Forward Primer 5' → 3'	Reverse Primer 5' → 3'	Amplicon Size (bp)	GenBank Accession Number
CysLT1	TCCTTAGAATGCAGAAGTCCGTG	AAATATAGGAGAGGGTCAAAGCAA	80	NM_001282187
ACTB	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA	139	NM_001101.3
HMBS	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC	154	NM_000190.3

2.7. Statistical Analysis

The data generated in this study was analyzed using the MedCalc software version 6.0 (Mariakerke, Belgium). To demonstrate statistical differences between the different stimulation conditions we used the Wilcoxon test for paired samples, which is a non-parametric alternative method for the paired-samples *t*-test when the distribution of the samples is not normal, *p* values equal or less than 0.05 was regarded as significant.

3. Results

3.1. Effect of LTC₄, LTD₄, and Cytokines on the Expression of CysLT1 Receptor

Nasal tissue fibroblasts from chronic rhinosinusitis patients were able to express CysLT1 receptor mRNA levels but at very low levels. Stimulation with LTC₄ did not influence the gene expression of the receptor when compared to non-stimulated cells; in contrast to LTD₄ that upregulated the mRNA levels of the receptor in a dose-dependent manner being 10^{−8} M the concentration with the highest effect (Figure 1).

Based on that fibroblasts may change their behaviour and activation profiles when they are under influence of certain cytokine milieu, we decided to study the effect of IL-4, IL-13, TNF-α and TGF-β₁ on the expression of CysLT1 receptor. Results of this stimulation showed that IL-4, IL-13, and TNF-α had no influence on the gene expression of CysLT1 receptor. In contrast, TGF-β₁ significantly increased the expression of the receptor when compared to unstimulated cells (Figure 2).

To evaluate the effect of cytokines on the changes induced by LTD₄ on CysLT1 receptor expression, chronic rhinosinusitis fibroblasts were pre-incubated 24 h with 10 ng/mL of TGF-β₁ and, subsequently, with different concentrations of LTD₄. Of interest, an additive effect on the regulation of CysLT1 receptor was observed. This effect was more pronounced at the LTD₄ concentration of 10^{−8} M as shown in Figure 3.

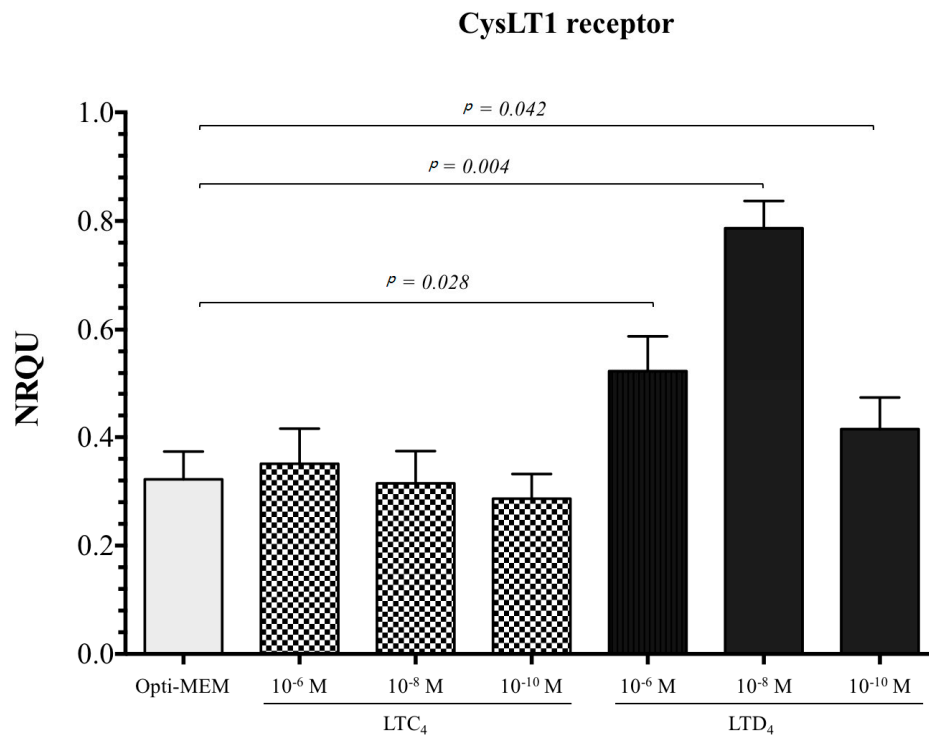


Figure 1. Messenger RNA expression levels of CysLT1 receptor in fibroblasts from chronic rhinosinusitis tissue after stimulation with cysteinyl leukotriene C₄ (LTC₄) and D₄ (LTD₄). NRQU: normalized relative quantification units; *p* value represents the level of significance after a Wilcoxon test for paired samples.

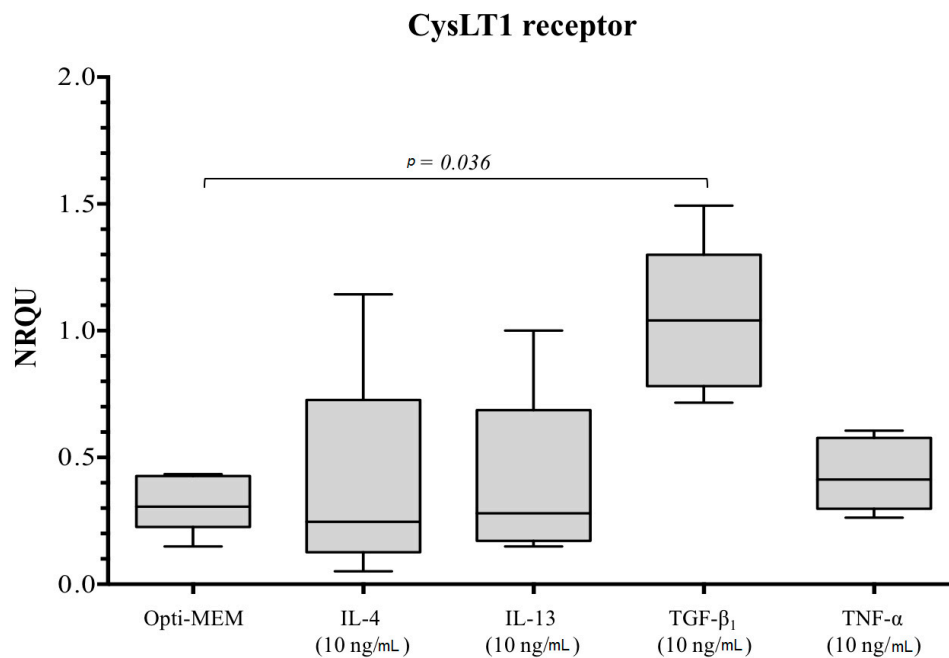


Figure 2. Messenger RNA expression levels of CysLT1 receptor in fibroblasts from chronic rhinosinusitis tissue after stimulation with Interleukin-4 (IL-4), interleukin-13 (IL-13), TNF-α, and TGF-β₁. NRQU: normalized relative quantification units; *p* value represent the level of significance after a Wilcoxon test for paired samples.

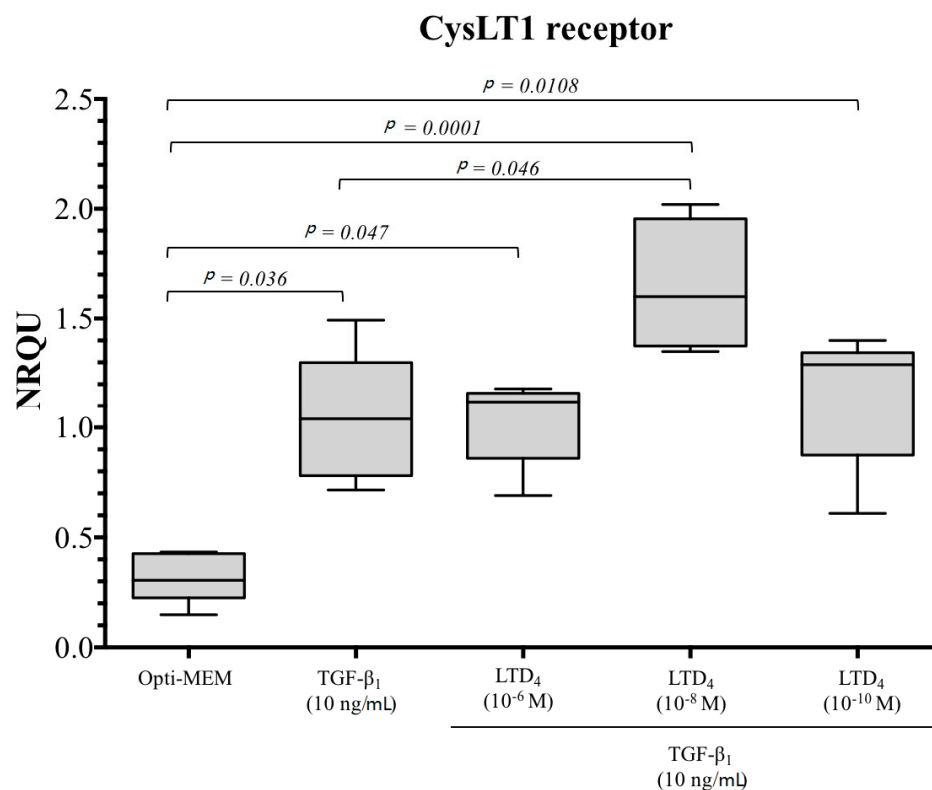


Figure 3. Messenger RNA expression levels of CysLT1 receptor in fibroblasts from chronic rhinosinusitis tissue after stimulation with TGF-β₁ and LTD₄. NRQU: normalized relative quantification units; *p* value represents the level of significance after a Wilcoxon test for paired samples.

3.2. Effect of LTC₄, LTD₄, and Cytokines on the Release of Metalloproteinases

Protein levels of MMP-2, MMP-3, MMP-7, and MMP-9 were below detection limit in all experimental conditions. Only MMP-1 and TIMP-1 were released during basal conditions. Further, LTD₄ increased the levels of MMP-1 in a dose-dependent way being the concentration of 10⁻⁸ M the one showing the best effect (Figure 4). No changes were observed in the expression of TIMP-1 by the addition of LTC₄ or LTD₄ in the culture medium.

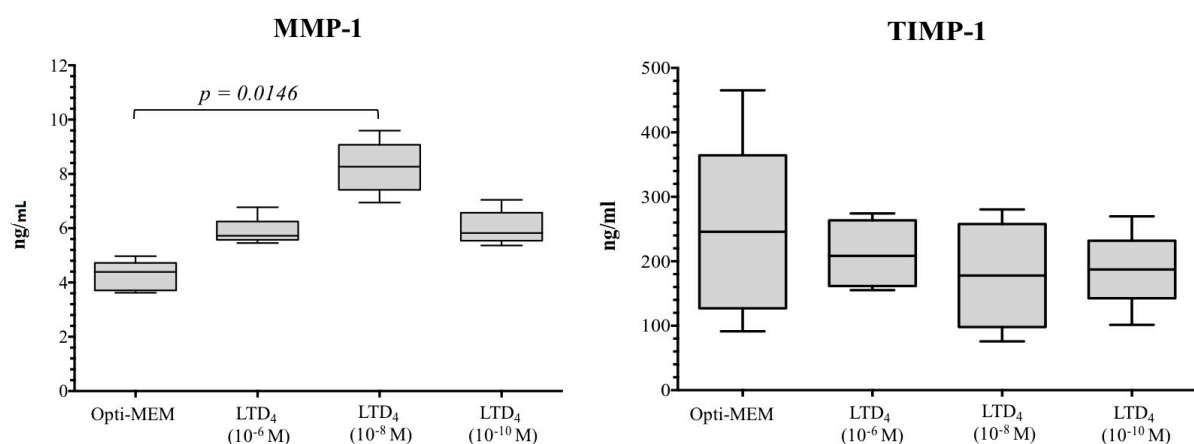


Figure 4. Concentrations of metalloproteinase 1 (MMP-1) and tissue inhibitor of metalloproteinase 1 (TIMP-1) in fibroblasts from chronic rhinosinusitis tissue after stimulation with LTD₄. *p* value represents the level of significance after a Wilcoxon test for paired samples.

Pre- incubation of the cells with IL-4, IL-13, TGF- β_1 , TNF- α alone or in combination with LTD₄ failed to induce changes in TIMP-1 production. Expression of MMP-1 was, however, significantly induced after TGF- β_1 and TNF- α stimulation alone and in combination with 10^{−8} M of LTD₄ when compared to non-stimulated cells (Figure 5). Addition of TNF- α increased the concentrations of MMP1 but this effect was not related to cytokine concentration, and a non-additive effect was observed after addition of LTD₄. The upregulation of MMP-1 levels by TGF- β_1 was more potent than the one observed with LTD₄ alone. An additive dose-dependent effect on MMP-1 production was obtained when combined with the two compounds in the culture (Figure 5).

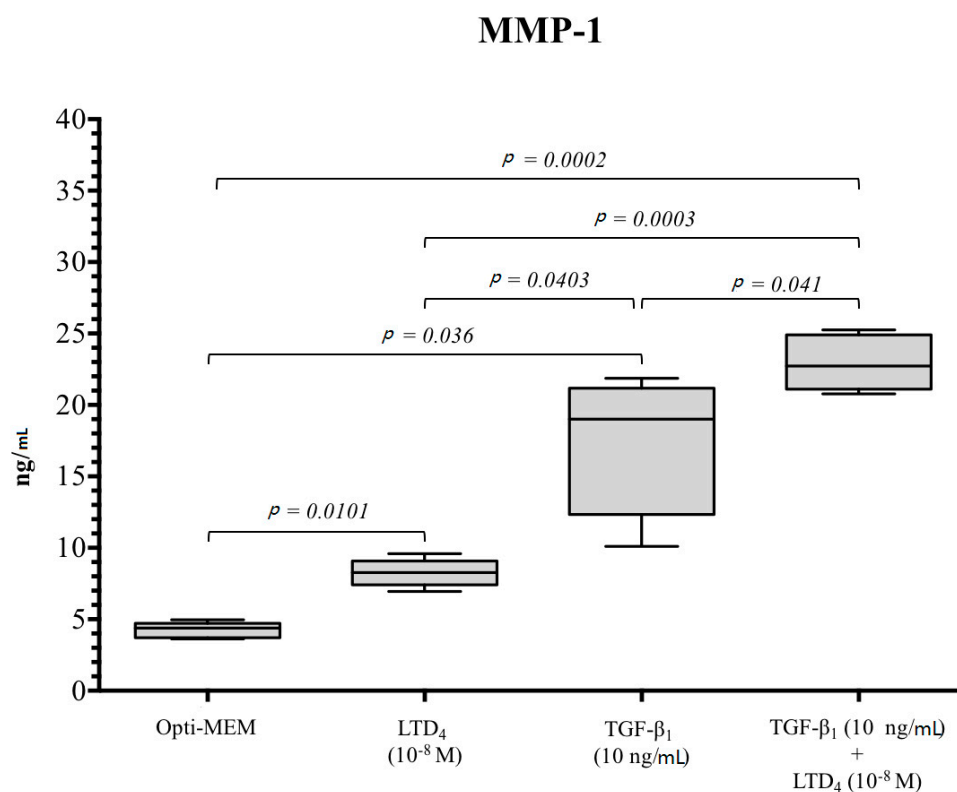


Figure 5. Concentrations of metalloproteinase 1 (MMP-1) in fibroblasts from chronic rhinosinusitis tissue after stimulation with LTD₄ and TGF- β_1 . *p* value represents the level of significance after a Wilcoxon test for paired samples.

3.3. Effect of Montelukast on MMP-1 and CysLT1 Receptor Expression Induced by LTD₄ and TGF- β_1

Montelukast inhibited the CysLT1 receptor mRNA expression in a dose-dependent manner in fibroblasts stimulated with LTD₄, but also attenuated the increase in receptor expression induced by TGF- β_1 alone and in combination with LTD₄ (Figure 6). The leukotriene receptor antagonist also reestablished in a dose-dependant way the concentrations of MMP1 to baseline levels in fibroblasts stimulated with TGF- β_1 and with LTD₄ alone or in combination. However, the lowest concentrations of Montelukast did not diminish the expression of MMP-1 to baseline levels in the cells pre-stimulated with TGF- β_1 (Figure 7).

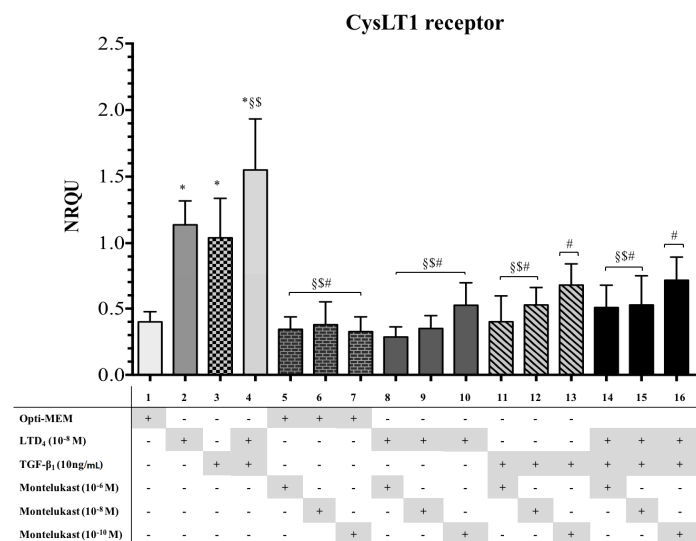


Figure 6. mRNA expression of CysLT1 receptor in fibroblasts from chronic rhinosinusitis tissue after stimulation with LTD₄ and TGF-β₁ and in presence or absence of Montelukast. NRQU: normalized relative quantification units, *: *p* value < 0.05 when compared to tissue culture medium (Opti-MEM), §: *p* value < 0.05 when compared to LTD₄ (10⁻⁸ M), \$: *p* value < 0.05 when compared to TGF-β₁, #: *p* value < 0.05 when compared to TGF-β₁ + LTD₄. Tukey's multiple comparisons test for CysLT1 receptor expression in Supplementary Table S1.

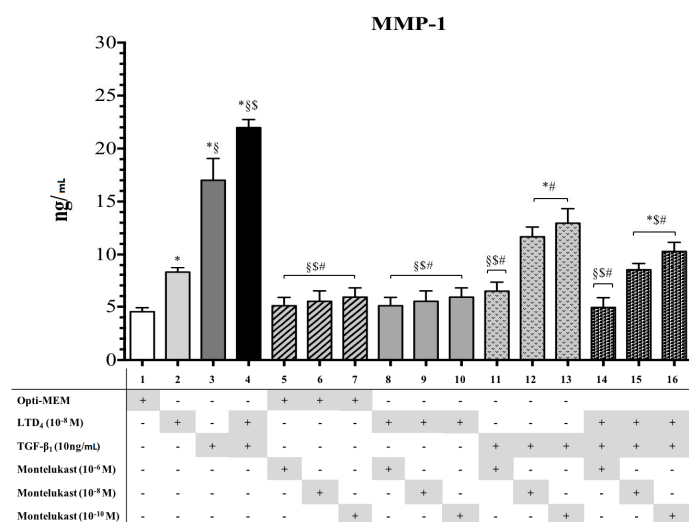


Figure 7. Concentrations of metalloproteinase 1 (MMP-1) in fibroblasts from chronic rhinosinusitis tissue after stimulation with LTD₄ and TGF-β₁ and in presence or absence of Montelukast. *: *p* value < 0.05 when compared to tissue culture medium (Opti-MEM), §: *p* value < 0.05 when compared to LTD₄ (10⁻⁸ M), \$: *p* value < 0.05 when compared to TGF-β₁, #: *p* value < 0.05 when compared to TGF-β₁ + LTD₄. Tukey's multiple comparisons test for MMP1 protein concentrations Supplementary Table S2.

4. Discussion

The main finding of this study is that TGF-β₁ and LTD₄ are potent inducers of MMP-1 protein in chronic rhinosinusitis tissue-derived fibroblasts and that this effect is mediated, in part, by the CysLT1 receptor and, hence, could be attenuated by its antagonist Montelukast. The effect of TGF-β₁ on MMP-1 production have been reported in lower airways; however, this is the first report in nasal

tissue fibroblasts. In CRSsNP tissue, MMP-1 levels are increased when compared to healthy nasal mucosa and it is mainly localized in the nasal epithelium and mucosal and sub-mucosal glands, as well as in some infiltrated inflammatory cells [27]. Further, TGF- β_1 and CysLTs are also increased in CRSsNP tissue when compared to non-inflamed nasal mucosa, as reported by Van Bruaene and Perez Novo *et al.*, respectively [12,28]. Balance in collagen degradation MMPs is a crucial mechanism leading to an appropriate tissue wound healing process where fibroblasts represent one of the major cells involved [2].

Leukotrienes play an important role in the regulation of MMP synthesis and, hence, in the remodeling process by enhancing collagenase mRNA expression [29]. Medina *et al.* demonstrated that LTD₄ and IL-1 β could induce the secretion of MMP-1 in airway smooth muscle cells resulting in the stimulation of cell proliferation [30]. More recently, it has been suggested that LTD₄ has the potential to augment fibroblast chemotaxis, and to contribute to the regulation of the wound healing and remodeling in fibrotic processes of the lung [31,32]. LTD₄ may also increase collagen production in activated myofibroblasts by upregulating CysLT1 receptor induced by TGF- β_1 [33]. This evidence strongly supports the possible role of lipid mediators, and especially cysteinyl leukotrienes, in airway remodeling and in the regulation of MMP production.

The action of TGF- β_1 through the CysLT1 receptor in the regulation of ECM molecules has been previously reported by Asakura *et al.* [33]. However, in that study LTD₄ in combination with TGF- β_1 stimulated the production of collagen in a human fetal lung fibroblasts cell line. Our results showed that the same compounds also induced the production of MMP-1, which can induce collagen degradation. This discrepancy can be due to the differences in cell type (lung derived cell line versus primary nasal tissue cells). As stated before MMP-1 degrades collagen. In a previous work we observed that collagen content in CRSsNP is higher than in control and CRSwNP tissues [28]. This is perfectly possible if we take into account that fibroblasts are not the only cells producing MMPs, and in our study we exclude the contribution of these cells. Further the expression of collagen and the influence of other cytokines, which are present in physiological conditions, could not be tested in this study.

It is important to mention that in the study of Steinke *et al.*, [34] the authors failed to show CyLT1 receptor expression in nasal polyp fibroblasts. One explanation for this discrepancy could be the high levels of TGF- β_1 present in CRSsNP tissue; this growth factor is an important inducer of CyLT1 receptor as demonstrated by several research works and observed in our study. This factor is also important in the transformation of fibroblasts into myofibroblasts. Fibroblasts isolated from CRSsNP could retain these properties during the seeding procedure and it may be the cause of the low expression of CyLT1 receptor observed. Furthermore, it has been documented from several authors that fibroblast behavior between CRS with and without polyps, and even between polyp subtypes, (for example, with and without asthma or aspirin-exacerbated disease) is different.

To conclude, we can state that the release of cytokines like TGF- β_1 and lipid mediators like CysLTs by inflammatory cells can significantly contribute to the regulation of fibroblast CysLT1 receptor and MMP-1 pathways. This is of relevance due to high levels of these molecules observed in chronic rhinosinusitis tissue. Montelukast is a potent and specific CysLT1 receptor inhibitor that has been shown to cause a significant decrease in sputum and peripheral blood eosinophil number, reduce concentrations of eosinophilic cationic protein, of LTC₄ and exhaled nitric oxide levels in asthmatic patients [35,36]. However, despite the action of leukotriene modifiers, it has been well documented that its role in upper airways remodeling remains unclear. The findings obtained in this study are in line with previous *in vitro* and animal studies, suggesting the use of Montelukast as a modifier of lower airway remodeling. Our work involved human nasal fibroblasts from chronic rhinosinusitis patients adding more evidence for the potential role of this LTR antagonist in regulating the remodeling process in upper airways.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2309-107X/1/1/65/s1>, Table S1: Tukey's multiple comparisons test for CysLT1 receptor expression. Table S2: Tukey's multiple comparisons test for MMP1 protein concentrations.

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Author Contributions: C.A.P.N. and C.B. conceived and designed the experiments; C.C., G.H. and C.A.P.N. performed the experiments; R.P. and C.A.P.N. analyzed the data; R.P., C.A.P.N. and C.B. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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