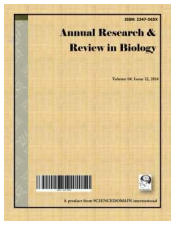

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CTLA4 Expression in Childhood Asthma and the Effect of Treatment with Inhaled Corticosteroid and Leukotriene Receptor Antagonist

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Authors' contributions

This work was carried out in collaboration between all authors. Author VB designed the study, interpreted the data and wrote the first draft of the manuscript. Author CEPK managed the literature searches, wrote the protocol, gathered the initial data and performed data analysis. Author UP designed the study, supervised the interpretation of the data and handled correspondence for the publication of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Cytotoxic T lymphocyte antigen 4 (CTLA4), an important regulatory molecule in the process of antigen presentation, was previously associated with the pathogenesis of autoimmune diseases and asthma. Therefore, the goal of our study was to determine the expression of *CTLA4* in

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asthmatics, and the effect of *CTLA4* CT60 genotype and antiasthmatic treatment on *CTLA4* expression.

Study Design: We analyzed a case-control cohort of 229 children with mild to moderate persistent asthma. Blood samples were collected before treatment from 229 asthmatics, with matching samples obtained 4-6 weeks after treatment with inhaled corticosteroid (ICS) in 69 subjects and after treatment with leukotriene receptor antagonist (LTRA) in 105 subjects.

Place and Duration of Study: Department of Pediatric Medicine, General Hospital Murska Sobota and University Medical Centre Maribor between January 2008 and May 2012.

Methodology: We measured and compared *CTLA4* expression in blood leukocytes of healthy controls and children with persistent asthma by qPCR and determined *CTLA4* CT60 genotype by High Resolution Melting analysis. We further analyzed how antiasthmatic treatment with ICS or LTRA affected *CTLA4* expression.

Results: Median relative expression of full length *CTLA4* (*flCTLA4*) isoform in asthmatics was 0.440±0.425, compared to 1.000±0.738 in controls (corrected *P*<.0001), and of soluble *CTLA4* (*sCTLA4*) isoform in asthmatics was 0.580±0.468 compared to 1.040±1.080 in controls (corrected *P*<.0001). After ICS therapy the median relative expression of *sCTLA4* significantly increased only in asthmatics with A allele, from 0.400±0.258 to 0.710±0.608 (corrected *P*=.0146). On the other hand, after LTRA therapy the median relative expression of *sCTLA4* decreased only in asthmatics with A allele from 0.450±0.410 to 0.300±0.300 (corrected *P*=0.0006).

Conclusion: We show that *CTLA4* expression is decreased in asthmatic subjects. ICS and LTRA treatments, dependent on *CTLA4* CT60 genotype, caused opposite effects on *sCTLA4* expression, suggesting both drugs differently affect molecular pathways of antigen presentation during their action.

Keywords: Antigen presentation; blood leukocytes; CTLA4 isoforms; CTLA4 CT60 polymorphism; antiasthmatic treatment; asthma pathogenesis.

ABBREVIATIONS

CTLA4 : Cytotoxic T lymphocyte antigen 4
qPCR : quantitatve polymerase chain reaction
ICS : Inhaled corticosteroid
LTRA : Leukotriene receptor antagonist
APC : Antigen presenting cell
flCTLA4 : full length cytotoxic T lymphocyte antigen 4
sCTLA4 : soluble cytotoxic T lymphocyte antigen 4
SNP : Single nucleotide polymorphism
CRHR1 : Corticotropin releasing hormone receptor 1
ORMDL3 : Orsomucoid1-like protein 3
GLCCI1 : Glucocorticoid induced transcript 1
LT : Leukotriene
PBMC : Peripheral blood mononuclear cells
IL : Interleukin
TNF : Tumor necrosis factor
NF-κB : Nuclear factor kappa B
GILZ : Glucocorticoid-induced leucine zipper)
FKBP51 : FK506-binding protein 51
ATS : American Thoracic Society
FVC : Forced vital capacity
FEV1 : Forced expiratory volume in first second
PC20 : Provocative concentration of methacholine causing a drop in FEV1 of 20%
IgE : Immunoglobulin class E
FENO : Fractional exhaled nitric oxide
Ppb : Parts per billion
EDTA : Ethylenediaminetetraacetic acid
DNA : Deoxyribonucleic acid

RNA : Ribonucleic acid
cDNA : complementary DNA
ACTB : Actin beta
B2M : Beta2-microglobulin
GAPDH : Glyceraldehyde-3-phosphate dehydrogenase
IQR : Interquartile range
3'UTR : Three prime untranslated region

1. INTRODUCTION

Asthma is a chronic inflammatory disease of airways of unknown etiology, characterized by hyper-responsiveness of the airways and reversible bronchial obstruction. Asthma is the most common serious chronic disease of childhood with a prevalence of around 10% before adolescence. Asthma pathogenesis is currently understood through the interaction of several genes and environmental influences [1].

CTLA4 is an immunoglobulin expressed on activated T cells. CTLA4 binds to B7-1 antigen on the antigen presenting cell (APC) and transmits an inhibitory signal to T cells in the process of antigen presentation by blocking the CD28-mediated co-stimulatory signal for the T-cell activation [2]. By limiting the APC-T-cell interaction, CTLA4-B7 pathway controls the delicate balance between allergic sensitization and tolerance, and appears to be one of the most important regulators of T cell responses to inhaled allergens in airways [3]. Human CTLA4 gene is mapped to chromosome 2q33. As a result of alternative splicing the CTLA4 protein exists in two isoforms of which the membrane bound full length (flCTLA4) isoform results from transcription of all the exons and is biologically more active, while the soluble isoform (sCTLA4) lacks the trans-membrane region [4].

A fine mapping association study in the *CTLA4* region revealed single nucleotide polymorphism (SNP) termed CT60 (rs3087243) to be significantly associated with different autoimmune diseases [5]. In addition, CT60 has been shown to influence the antiasthmatic effect of ICS [6].

ICSs are currently the mainstay of the anti-asthmatic treatment [7]. They are however, ineffective in almost a quarter of properly treated asthmatics, and the differences in anti-asthmatic effect between individuals are caused by several factors, including microbiota and genotype [8]. So far, only a few genes have been associated with the effect of ICS in asthma, among them are genes for corticotropin releasing hormone receptor 1 (CRHR1), glucocorticoid receptor and ORM1-like protein 3 (ORMDL3) [9-11]. The first genome wide association study in this field revealed the influence of glucocorticoid induced transcript 1 (GLCCI1) gene on the effect of ICS in childhood asthma [12]. Corticosteroids negatively influence the antigen presentation and T-cell activation, however, the exact molecular mechanism of their anti-asthmatic action is still not known [13].

ICS inhibit numerous airway inflammatory cells and mediators that are pivotal in the asthma pathogenesis, whereas LTRA selectively block leukotriene (LT)-mediated eosinophilic inflammation. The anti-inflammatory effects of ICS are undoubtedly broader compared to LTRA however, LTRA also exert wide control over the asthmatic inflammation, partially via indirect inhibitory effects on the synthesis or actions of pro-inflammatory cytokines [14]. LTRA are antagonists of cysteinyl leukotriene 1 receptor which promotes most of the pathophysiological effects of leukotrienes in asthma, including increased airway smooth muscle activity, microvascular permeability, and airway mucus secretion [15]. As the LT pathway is relatively resistant to treatment with ICS, LTRA may contribute to asthma treatment [16].

The aim of this study was to determine the CTLA4 gene expression in asthmatic subjects and correlate gene expression data to ICS and LTRA treatment response.

2. MATERIALS AND METHODS

2.1 Patients and Study Design

We analyzed a case-control cohort composed of 229 children with mild or moderate persistent asthma, aged 5-18 years of which 142 were atopic asthmatics, 74 were non-atopic asthmatics, and 13 patients with undetermined atopy status, and 92 healthy unrelated but age and sex matched controls. Asthma was diagnosed according to American Thoracic Society (ATS) criteria [17,18]. Subjects were stratified into two groups based on the results of allergy testing (skin prick test and

specific IgE values), which was performed before the stratification. Subjects who had positive test to at least one aeroallergen were stratified into atopic asthmatic group. Subjects with negative tests to aeroallergens were stratified into non-atopic asthmatic group. Age, sex and asthma severity of both groups were matched. Allergy testing was not successful in 13 subjects (lack of response to histamine, demographics or skin inflammation and borderline specific IgE values), who were subsequently not stratified. Results were analyzed and presented in the whole group of asthmatics and separately in atopic and non-atopic asthma group. Asthmatics were treated in the Pulmonary and Allergic Outpatient consultation, Department of Pediatric Medicine, General Hospital Murska Sobota and in the Pediatric Pulmonary Outpatients, University Medical Centre Maribor from 01.01.2008 to 31.05.2012. We included all children with mild to moderate persistent asthma newly diagnosed in this period, who had no antiasthmatic treatment, except the short acting beta2 agonists occasionally as relief medication. All subjects and controls were Caucasians of Slovenian origin. Patients with other chronic inflammatory diseases except atopic diseases associated with asthma were excluded from the study. All measurements, blood withdrawal and laboratory tests were done before the initiation of treatment and when the subjects were without any acute disease. For gene expression analysis, blood samples were collected before treatment from 229 asthmatics. Matching samples were taken 4-6 weeks after the initiation of treatment, however only in those subjects who agreed with a second blood withdrawal. Both group of asthmatics were further stratified according to treatment – 69 (48 atopics and 20 non-atopics) of them were treated with ICS and 105 (63 atopics and 38 non-atopics) with LTRA. Comparison of pre- and post-treatment expression refers only to matched samples. In ICS treatment group 200 µg of fluticasone dry powder (Flixotide diskus®, GSK Pharmaceuticals S.A., Posen, Poland) per day was prescribed for children younger than 12 years of age and 400 µg daily for older children. In LTRA treatment group subjects were treated with montelukast (Singulair®, Merck&Co., Inc., Whitehouse station, NJ, USA) – 5 mg tablets were prescribed for children under 12 years old and 10 mg tablets for older children.

This study was approved by the Slovenian National Medical Ethics Committee (KME 31/12/06) and carried out in accordance with the Helsinki declaration of the World Medical Association (1975). Parents signed informed consent for children younger than 15 years old, while older children gave informed consent by themselves.

2.2 Measurements and Laboratory Tests

Several important clinical and laboratory parameters were measured in asthmatics and handled as quantitative variables: forced vital capacity (FVC), forced expiratory volume in 1 sec expressed as a percent of predicted value for sex, height and age before treatment (FEV1 b.t.), after treatment with ICS (FEV1 a.t.), difference of both (ΔFEV1 = FEV1 a.t. minus FEV1 b.t.), FEV1/FVC ratio, the provocative concentration of methacholine causing a drop in FEV1 of 20% (PC20) and its base 10 logarithm (logPC20), total immunoglobulin class E (IgE) concentration, eosinophil count in peripheral blood and fractional exhaled nitric oxide (FENO) measured in parts per billion (ppb). Allergic status was determined with the skin prick tests (Allergopharma, Reinbek, Germany) and specific IgE to the most common aeroallergens (CAP-RAST Pharmacia&Upjohn, Freiburg, Germany). All clinical and laboratory parameters were measured as previously described [11].

In asthmatics who were treated with ICS or LTRA, spirometry was repeated after 4-6 weeks of treatment and the ΔFEV1 value was used as a measure of treatment outcome.

Twelve milliliters of venous blood was drawn from each patient into tubes with EDTA for genetic analysis, an eosinophil count and total IgE analysis.

2.3 DNA and RNA Extraction

Total blood leukocytes were isolated using Ficoll-Paque® Plus (GE Healthcare, Uppsala, Sweden) gradient centrifugation, according to the manufacturer's instructions. Total RNA and genomic DNA were isolated using QIAzol® Lysis Reagent (QIAgen, Valencia, CA, USA). DNA was dissolved in water at final concentration of 50 ng/µl. RNA concentrations ranged from ~0.1–1.17 µg/µL as determined by an ND1000 spectrophotometer and NanoDrop® 3.0.1 software (NanoDrop Technologies, Wilmington, DE, USA); 260/280 ratios ranged from 1.7 to 2.0. The integrity of RNA samples was analyzed by electrophoresis on a 2% agarose gel. All

samples were immediately frozen and stored at -80℃.

2.4 Genotyping of Polymorphism CT60

Genotyping was performed by High Resolution Melting (HRM) curve analysis following touchdown PCR amplification. Primers used for touchdown PCR amplification were designed using Primer3 (http://simgene.com/Primer3), manufactured by Sigma (Steinheim, Germany) and the sequence was: Forward 5´-TCCATCCTCTTTCCTTTTGA and reverse 5´-AAACAGCATGCCAATTGATTT. The touchdown PCR amplification was performed using a 96 multiwell white-plate (Cat.#04729692001, Roche Applied Science, Mannheim, Germany) on a Roche LightCycler® 480 detection system (Roche Applied Science, Mannheim, Germany). Samples were amplified in reactions containing 2 µL of genomic DNA (2.5 ng/µL), 3 µL of 2x LightCycler® 480 High Resolution Melting Master mix (Roche Applied Science, Mannheim, Germany), 0.061 µL of each primer (200 nM final concentration), 0.72 µL of MgCl2 (3 mM final concentration), and RNase-free water in a final reaction volume of 6 µL. The touchdown PCR program was initiated at 95℃ for 10 min, followed by 45 thermal cycles of 10 sec at 95℃, 15 sec at 63℃ (secondary target temperature 53℃, with 0.5℃ steps) and 10 seconds at 72℃. The HRM curve analysis was performed with a temperature range used for the melting curve generation from 65℃ to 95℃ with 25 signal acquisitions per ℃.

DNA samples not passing high-quality control standards were excluded from genotyping which, resulted in the exclusion of 5 DNA samples from asthmatic subjects and 9 from control subjects.

2.5 Gene Expression Measurements

First-strand cDNA was generated by reverse transcription of 1 µg total RNA per sample with random primers and MultiScribeTM Reverse Transcriptase (50U/reaction) using High-Capacity cDNA Reverse Transcription kit (Cat. #4368813, Applied Biosystems, Foster City, CA, USA) in a final reaction volume of 20 µL as previously described [19].

The intron spanning primers used for reference genes *ACTB*, *B2M* and *GAPDH*, amplification were designed using the Universal ProbeLibrary Assay Design Center from Roche Applied Science (https://www.roche-applied-science.com/sis/rtpcr/upl) and manufactured by Sigma (Steinheim, Germany).

We measured expression of both *CTLA4* isoforms (*sCTLA4* and *flCTLA4*) before and after 4-6 weeks of anti-asthmatic treatment with ICS or LTRA using previously published primers for specific quantification of *sCTLA4* and *flCTLA4* isoform expression [20]. The expression study was performed by qPCR as previously described [19]. Briefly, samples were amplified in reactions containing 2 µL of cDNA, 5 µL of 2x SYBR Green master mix, primers (concentration according to optimized standard curve of each gene) and RNase-free water in a final reaction volume of 10 µL. *flCTLA4* and *sCTLA4* Cq values were normalized using the geometrical mean of reference genes *ACTB*, *B2M* and *GAPDH* Cq values [19]. Relative expression was calculated using the equation $2^{-\Delta\Delta Cq}$, where $\Delta\Delta Cq = -(Cq_{target} - Cq_{geometrical\ mean\ reference})$ sample $-$ Average $(Cq_{target} - Cq_{geometrical\ mean\ reference})$ control.

RNA samples not passing high-quality control standards, or with Cq values above 35, were excluded from gene expression analysis. From *flCTLA4* gene expression analysis 45 samples from the asthmatic subjects group before treatment were excluded, 16 samples from asthmatic subjects after ICS treatment, 31 samples from asthmatic subjects after LTRA treatment and 16 samples from control group. From *sCTLA4* gene expression analysis 77 samples from the asthmatic subjects group before treatment were excluded, 22 samples from asthmatic subjects after ICS treatment, 46 samples from asthmatic subjects after LTRA treatment and 17 samples from control group.

2.6 Statistical Analysis

Data analysis was carried out using SPSS® version 19.0 (SPSS, Chicago, IL, USA). When comparing expression of *CTLA4* between asthmatics and controls we used Kruskal–Wallis test followed by correction for multiple comparisons with Dunn's post hoc test. Influence of CT 60 genotype on *CTLA4* gene expression was assessed with Kruskal–Wallis test followed by Dunn's multiple comparison test and was confirmed with generalized linear model adjusting for age and gender.

The correlation between clinical parameters and *CTLA4* gene expression was analyzed by Spearman's correlation coefficient. The influence

of anti-asthmatic treatment on *CTLA4* gene expression was assessed with Wilcoxon signed-rank test. Data are presented as median± interquartile range (IQR), and *P*-value of less than 0.05 were considered to indicate statistical significance. Bonferroni corrected *P*-values are indicated by *P*c.

3. RESULTS

3.1 Genotype Frequencies of SNP CT6

The CT60 (rs3087243) genotype frequencies for the asthmatic subjects in this study were 36.6% (n = 82), 51.3% (n = 115) and 12.1% (n = 27) for A/A, A/G and G/G genotype, respectively. Genotyping was unsuccessful in 5 subjects. For the control group CT60 genotype frequencies were 42.5% (n = 37), 37.9% (n = 33) and 19.5% (n = 17) for A/A, A/G and G/G genotype, respectively. Genotype frequencies were not significantly different between asthmatics and controls (A/A vs. A/G+G/G, *P* = .36; G/G vs. A/G+A/A, *P* = .11). Genotype frequencies in the group of asthmatics and in the control group were in Hardy–Weinberg equilibrium (*P* = .62 and *P* = .61, respectively).

3.2 Expression of *sCTLA4* and *flCTLA4* Isoforms

Median relative expression of *sCTLA4* isoform in asthmatics before the treatment was significantly lower (0.580±0.468) compared with controls (1.040±1.080) (P_c < .0001). In atopic asthmatics *sCTLA4* median relative expression was 0.450±0.395 and in non-atopic asthmatics was 0.425±0.490 which were both significantly lower when compared with controls (P_c < .0001) (Fig. 1A).

Median relative expression of *flCTLA4* isoform in asthmatics before anti-asthmatic treatment was significantly lower (0.440±0.425) compared with controls (1.000±0.738) (*Pc* < .0001). Median relative expression of *flCTLA4* in atopic (0.450±0.395) and non-atopic (0.425±0.490) asthmatics was also found to be significantly lower (*Pc* < .0001) compared with controls. The results are shown in Fig. 1B.

Considering only the subjects with matched samples (who agreed with second blood withdrawal) the median relative expression of *sCTLA4* after ICS treatment significantly increased from 0.440±0.320 to 0.680±0.565 in asthmatics (*P* = .04, *Pc* = .07) and from 0.430±0.340 to 0.710±0.530 in atopic asthmatics (*P* = .02, *Pc* = .049) (Fig. 2A). In non-atopic asthmatics no significant change was found in the *sCTLA4* median relative expression after treatment with ICS.

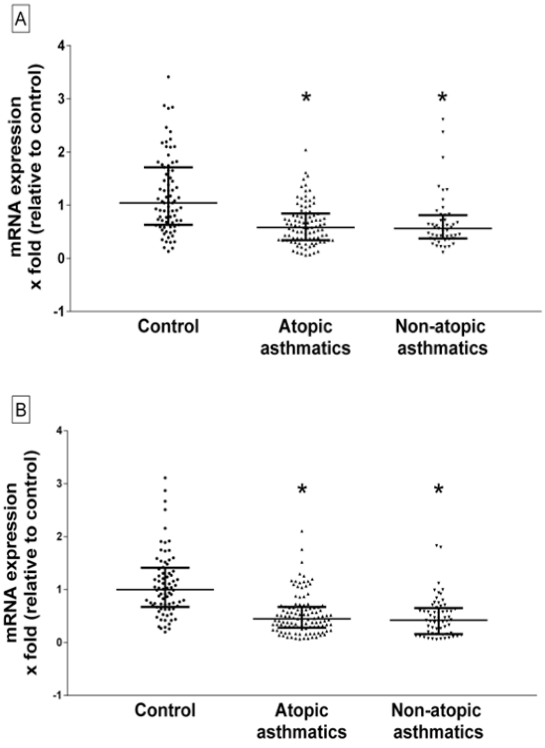

Fig. 1. CTLA4 gene expression in asthmatic subjects before treatment

*A - sCTLA4 gene expression; Control (n = 80), Atopic asthmatics (n = 113) and Non-atopic asthmatics (n = 60). B – flCTLA4 gene expression; Control (n = 79), Atopic asthmatics (n = 95) and Non-atopic asthmatics (n = 47). Lines represent median gene expression and error bars illustrate the interquartile range. *P < .0001 compared with 'Control' (Kruskal-Wallis followed by Dunn's multiple comparison test)*

After LTRA treatment the median relative expression of *sCTLA4* significantly decreased from 0.440±0.520 to 0.310±0.355 in asthmatics (*P* = .007, *Pc* = .02) and from 0.640±0.690 to 0.330±0.315 in non-atopic asthmatics (*P* = .006, *Pc* = .02) (Fig. 2B). No significant change in the median relative expression of *sCTLA4* was found in atopic asthmatics.

After ICS treatment the median relative expression of *flCTLA4* in asthmatics did not changed significantly compared to median relative expression before treatment (Table 1).

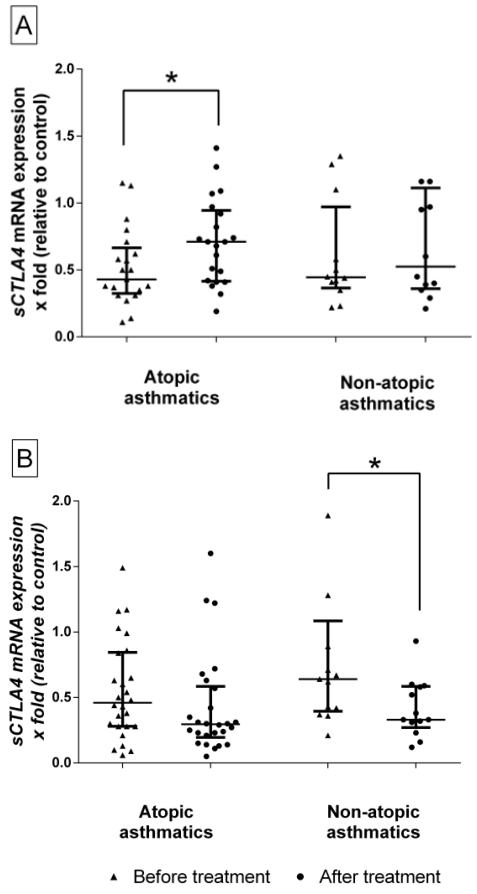

Fig. 2. Comparative expression of sCTLA4 in matching asthmatic subjects before and after treatment

*A – After inhaled corticosteroid treatment; Atopic asthmatics (n = 21) and Non-atopic asthmatics (n = 12). and B – After leukotriene receptor antagonist treatment; Atopic asthmatics (n = 26) and Non-atopic asthmatics (n = 13). Lines represent median gene expression and error bars illustrate the interquartile range. *P < .05, Wilcoxon matched-pairs signed rank test*

After LTRA treatment no significant changes in the median relative expression of *flCTLA4* were found in asthmatics or any of asthma subgroups (Table 1).

3.3 Correlation of *CTLA4* Expression with Clinical Data and Treatment Outcome

Positive correlation of *sCTLA4* expression before treatment with total IgE values was found in non-atopic asthmatics, however, after Bonferroni correction this correlation was not found to be significant (*P* = .012, *Pc* = .072) (Table 2). No other correlations of *CTLA4* expression before treatment were found with clinical data.

3.4 Influence of CT60 Genotype on *CTLA4* Expression

No significant association of CT60 genotype on *flCTLA4*, *sCTLA4* median relative expression before treatment was found in asthmatics and control subjects (Table 3).

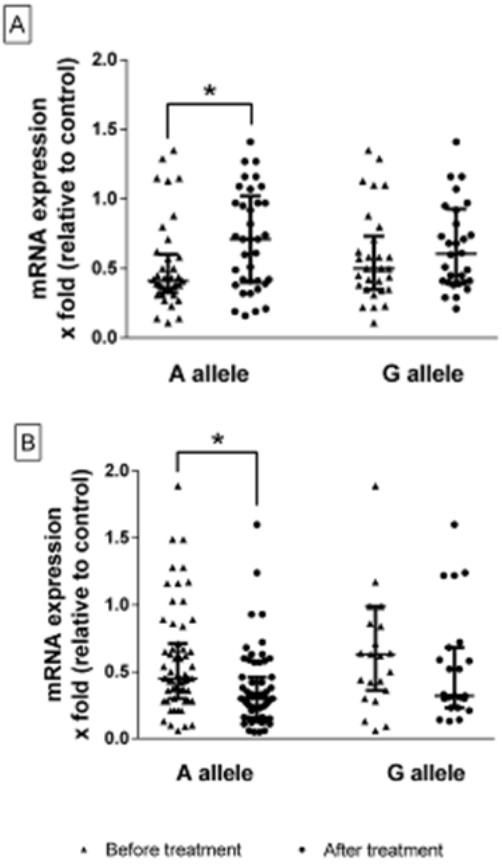

Fig. 3. Comparative expression of sCTLA4 in matching asthmatic subjects before and after treatment according to CTLA4 CT60 genotype

*A – After inhaled corticosteroid treatment; A allele (n = 37) and G allele (n = 30). B – After leukotriene receptor antagonist treatment; A allele (n = 59) and G allele (n = 23). Lines represent median gene expression and error bars illustrate the interquartile range. *p < 0.05, Wilcoxon matched-pairs signed rank test*

We confirmed these results with generalized linear model analysis (*P* = .10 for *flCTLA4* and *P* = .19 for *sCTLA4*).

Table 1. *CTLA4* gene expression in asthma patients after treatment with inhaled corticosteroid (ICS) or leukotriene receptor antagonist (LTRA)

	ICS treatment						LTRA treatment					
	flCTLA4						*flCTLA4*					
	Before treatment		After treatment		*n*	*P*	Before treatment		After treatment		*n*	*P*
	Median	IQR	Median	IQR			Median	IQR	Median	IQR		
Asthmatics	0.390	0.500	0.545	0.638	42	.26	0.390	0.430	0.330	0.390	63	.44
Atopic asthmatics	0.345	0.468	0.575	0.633	26	.06	0.380	0.385	0.310	0.350	41	.15
Non-atopic asthmatics	0.595	0.665	0.380	0.820	16	.90	0.430	0.515	0.360	0.605	21	.56

*flCTLA4, full length CTLA4 isoform; IQR, interquartile range. * P < .05 considered statistically significant (Wilcoxon sign-ranked test)*

Table 2. *CTLA4* gene expression correlation with the clinical data before treatment

	Asthmatics			
	flCTLA4		*sCTLA4*	
	r	*P*	*r*	*P*
FEV1/FVC	0.042	.57	0.039	.64
FEV1	-0.117	.114	-0.085	.30
logPC_{20}	-0.045	.55	-0.115	.16
FENO	-0.044	.56	-0.037	.66
Total IgE	0.161	.30	0.216	.17
Eosinophilia	-0.004	.98	0.035	.83
	Atopic asthmatics			
	flCTLA4		*sCTLA4*	
	r	*p*	*r*	*p*
FEV1/FVC	0.018	.85	-0.023	.83
FEV1	-0.135	.15	-0.120	.25
logPC_{20}	-0.088	.35	-0.139	.18
FENO	0.040	.68	-0.001	.99
Total IgE	0.348	.08	0.168	.41
Eosinophilia	0.16	.449	0.061	.77
	Non-atopic asthmatics			
	flCTLA4		*sCTLA4*	
	r	*P*	*r*	*P*
FEV1/FVC	0.039	.77	0.097	.52
FEV1	-0.093	.48	0.072	.63
logPC_{20}	0.098	.45	-0.054	.72
FENO	-0.235	.08	0.030	.85
Total IgE	0.437	.12	0.647	.01*
Eosinophils	0.077	.80	0.055	.86

*flCTLA4, full length CTLA4 isoform; sCTLA4, soluble CTLA4 isoform; FEV1, forced expiratory volume, 1 s; FVC, forced vital capacity; logPC20, provocative concentration of methacholine; FENO, fractional exhaled nitric oxide; IgE, immunoglobulin class E; r, Spearman's correlation coefficient, *P < .05 considered statistically significant*

We also tested the influence of CT60 genotype on the change of *sCTLA4* expression after the treatment (Fig. 3), since this was the only isoform whose expression was significantly changed after the treatment. After ICS treatment the median relative expression of *sCTLA4* significantly increased in asthmatics with A allele from 0.410±0.275 to 0.710±0.620 (*P* = .007, *Pc* = .01), but not in asthmatics with G allele (*P* = .19). On the other hand, after LTRA treatment the median relative expression of *sCTLA4* significantly decreased in asthmatics with A allele from 0.450±0.410 to 0.300±0.300 (*P* = .0003, *Pc* = .0006), but not in asthmatics with G allele (P = .14).

4. DISCUSSION

We found a significantly lower expression of both isoforms; *sCTLA4* (soluble) and *flCTLA4* (full length, membrane bound), in all asthmatics as well as in atopic and non-atopic asthma subgroups analyzed separately. We also found that after treatment with ICS, *sCTLA4* isoform expression increased significantly in asthmatics and in the subgroup of subjects with atopic asthma, but only a non-significant trend in increase was observed in non-atopic asthmatics. In contrast, after treatment with LTRA, *sCTLA4* expression significantly decreased in asthmatics and non-atopic asthma subgroup.

To the best of our knowledge our study is the first to report the effect of LTRA treatment on *CTLA4* expression in asthmatic subjects. Moreover, so far there have not been any studies analyzing the effect of LTRA on gene expression in humans. The observed opposite effect of ICS and LTRA on *CTLA4* expression further illustrates how different drugs differently affect molecular pathways.

ICS have broad spectrum of anti-inflammatory effects and they also inhibit the antigen presentation and T cell activation – a process where CTLA4 molecule also has an important regulatory role [21]. ICS however do not affect leukotriene synthesis in asthma and moreover, proinflammatory effects of cysteinyl-leukotrienes are resistant to ICS [22]. Cysteinyl leukotrienes are produced in the airways during respiratory infection and beside the stimulation of airway smooth muscle activity, microvascular permeability and airway mucus secretion, they are also involved in the protection against

respiratory pathogens, probably by affecting the migration and functions of APC [23]. Recent clinical studies and meta-analysis from Cochrane Database showed that the antiasthmatic effect of LTRA is inferior to ICS in most asthma phenotypes [24,25]. Furthermore, corticosteroids suppressed pulmonary APC in mice asthma model, whereas LTRA had no effect on APC in allergic mice and even up-regulated APC in respiratory syncytial virus infected and non-sensitized mice [26]. Our results indicate the different or even opposite effect of LTRA (compared to ICS) on antigen presentation, especially in non-atopic asthma, where viral infections play more important role. We suggest that ICS inhibit antigen (allergen) presentation in atopic asthma and LTRA stimulate (viral) antigen presentation in non-atopic asthma. Up-regulation of viral antigen presentation by LTRA could have a beneficial effect in viral elimination and thereby non-atopic asthma treatment. Up-regulation of antigen presentation represented by increase in *sCTLA4* expression could be one of the important mechanisms of action of LTRA in viral induced wheezing, where they are more successful compared to atopic asthma treatment [27]. The increase of *sCTLA4* expression after ICS treatment in atopic asthmatics could be explained by the greater importance of allergen presentation in the atopic asthma pathogenesis compared with non-atopic asthma where viral infections are the most common trigger [28]. Increase in expression of *CTLA4* after the treatment with ICS observed in our study is in line with the finding of Kawayama et al. who also found an increase of expression in the sputum cells of asthmatics after the treatment with ICS [29].

When stratifying for *CTLA4* CT60 genotype (rs3087243), we found a significant increase in *sCTLA4* expression after ICS treatment in carriers of A allele compared to carriers of G allele of the same SNP. We found in our previous study association between A allele carriers and better anti-asthmatic effect of ICS [6] however, no correlation was found in the present study between increase of *sCTLA4* expression itself and treatment outcome. SNP CT60 in 3′UTR is in linkage disequilibrium with the more frequently studied polymorphisms 49A/G in the 5′UTR and 318C>T in the promoter region of *CTLA4* gene which were previously shown to influence *CTLA4* expression in lymphocytes from healthy individuals [5]. Variants associated with increased expression of *CTLA4* were found to protect against some autoimmune diseases and promote the emergence of others, indicating the complex effect of CTLA4 on the immune system [30,31]. Furthermore, genotypes such as 49A/A which protect against autoimmune diseases were found to be associated with atopy [5]. *CTLA4* 49A/A and 318T/T genotypes were shown to be associated with atopic asthma or asthma severity in Korean and Japanese children [32,33]. Although we found correlation between *CTLA4* genotype CT60 and increase in *sCTLA4* expression after ICS treatment, we did not observe any effect of *CTLA4* CT60 genotype on *flCTLA4* or *sCTLA4* expression levels before treatment.

Lack of correlation of CTLA4 expression with clinical characteristics (except total IgE) observed in our study is probably explained by homogeneity of our participants regarding the clinical characteristics, as we excluded patients with severe and mild intermittent asthma.

Our study is also the first to report altered *CTLA4* isoform's expression in human blood lymphocytes from asthmatic subjects, which are easier to collect than lower airways specimen and share most abnormalities with T cells in bronchial mucosa [34]. Interestingly, the expression of *CTLA4* gene was found to be up-regulated in inflamed tissue [35] and in blood lymphocytes [36] from autoimmune and chronic inflammatory diseases such as Crohn's disease and ulcerative colitis. The lowered expression of *CTLA4* in asthmatics found in the present study also suggests a different biological role of CTLA4 in autoimmune diseases compared with asthma and an inhibitory influence of both CTLA4 isoforms on the Th2 immune response, a key element in atopic asthma pathogenesis. Th2 immune response is of less significance in autoimmune diseases, where Th1 immune response predominates [37]. CTLA4 inhibits the Th1 and Th2 subtype activation, but its expression is much higher on Th2 compared to Th1 cells [38].

The functional consequences for mRNA levels of CTLA-4 and correlation between mRNA and protein levels were already proven elsewhere [5]. Therefore, we did not find it necessary to perform protein analysis to provide additional confirmation. Moreover, several published papers already described very well the correlation between total *CTLA4* mRNA levels and proteins [39,40], including the soluble isoform [41], which is particularly relevant for our study since our major conclusions are derived from measurements of sCTLA4 isoform.

Table 3. Influence of CT60 genotype on CTLA4 expression in asthmatics before treatment and control subjects

	AA			AG			GG			AA vs. AG vs. GG	AG+GG			AA vs AG+GG	AG+AA			GG vs. AG+AA
	Median	**IQR**	***n***	**Median**	**IQR**	***n***	**Median**	**IQR**	***n***	***P***	**Median**	**IQR**	***n***	***P***	**Median**	**IQR**	***n***	***P***
flCTLA4	0.570	0.538	96	0.530	0.650	127	0.620	0.851	36	.80	0.570	0.680	163	.83	0.550	0.600	223	.51
sCTLA4	0.705	0.656	90	0.640	0.705	105	0.690	0.570	31	.99	0.645	0.675	136	.96	0.670	0.670	195	.99

flCTLA4, full length CTLA4 isoform; sCTLA4, soluble CTLA4 isoform

5. CONCLUSION

We have confirmed that *CTLA4* expression is down-regulated in atopic and non-atopic asthmatics. ICS and LTRA treatments caused opposite effect on *sCTLA4* expression suggesting both drugs differently affect molecular pathways of antigen presentation during their action in asthmatics. The effect of ICS and LTRA on *CTLA4* expression depends on *CTLA4* CT60 genotype. Further studies are needed for identification of molecular biomarkers for personalized treatment of asthmatics.

CONSENT

All authors declare that 'written informed consent was obtained from the patients (or other approved parties) for publication of these data.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the Slovenian National Medical Ethics Committee (KME 31/12/06) and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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