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Budesonide increases TLR4 and TLR2 expression in Treg lymphocytes of allergic asthmatics

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ABSTRACT

Background: Reduced innate immunity responses as well as reduced T regulatory activities characterise bronchial asthma.
Objectives: In this study the effect of budesonide on the expression of TLR4 and TLR2 in T regulatory lymphocyte sub-population was assessed.
Methods: TLR4 and TLR2 expression in total peripheral blood mononuclear cells (PBMC), in CD4+/CD25+ and in CD4+/CD25− was evaluated, by flow cytometric analysis, in mild intermittent asthmatics ($n = 14$) and in controls ($n = 11$). The *in vitro* effects of budesonide in modulating: TLR4 and TLR2 expression in controls and in asthmatics; IL-10 expression and cytokine release (IL-6 and TNF-α selected by a multiplex assay) in asthmatics were also explored.
Results: TLR4 and TLR2 were reduced in total PBMC from asthmatics in comparison to PBMC from controls. CD4+CD25+ cells expressed at higher extent TLR2 and TLR4 in comparison to CD4+CD25− cells. Budesonide was able to increase the expression of TLR4, TLR2 and IL-10 in CD4+/CD25highly+ cells from asthmatics. TLR4 ligand, LPS induced Foxp3 expression. Budesonide was also able to reduce the release of IL-6 and TNF-α by PBMC of asthmatics.
Conclusions: Budesonide potentiates the activity of Treg by increasing TLR4, TLR2 and IL-10 expression. This event is associated to the decreased release of IL-6 and TNF-α in PBMC treated with budesonide. These findings shed light on new mechanisms by which corticosteroids, drugs widely used for the clinical management of bronchial asthma, control T lymphocyte activation.
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1. Introduction

Asthma is a heterogeneous disorder that is characterized by variable and largely reversible airflow obstruction, airway inflammation and hyperresponsiveness. Airway inflammation in allergic asthma is characterized by exaggerated activation of T helper type-2 (Th2) cells, IgE production and eosinophilia [1]. An increased survival of immune effector cells (eosinophils, macrophages and T lymphocytes) within the airways contributes to the severity of asthma [2]. T-lymphocytes play a crucial role in the development of airway inflammation. Particularly, Tregs exert an important role in the control of T-cell-mediated inflammation in asthma [3,4]. Two major subsets of Tregs are identified: CD25highly + Foxp3+ Tregs and IL-10-producing Tregs. The numbers or function of both Treg subsets are deficient in patients with atopic allergic diseases including asthma [5]. Enhanced production of IL-6 induces the increase of allergic inflammation via suppression of regulatory T-cells [6]. In this regard, it has been demonstrated that the pro-inflammatory cytokine IL-6 inhibits the TGFβ-induced generation of Foxp3 Treg [7].

Several epidemiological studies shed light on the possible protective influences of natural microbial exposure on atopy and

Abbreviations: BUD, budesonide; FEV1, forced expiratory volume in 1 s; TLR, toll like receptor; Treg, T regulatory lymphocytes; LPS, lipolysaccharide; LPT, lipoteichoic acid.

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asthma development and have led to the formulation of the hygiene hypothesis [8]. According to this hypothesis, a constant Th1 triggering balances the immune system, and the removal of these triggers skews the system toward Th2. The lack of Th1-inducing factors, i.e. the reduction of pathogen-associated molecular patterns (PAMP), which are detected by cells expressing Toll-like receptors (TLRs), is associated with the germ-reduced living standard in the cities of the so-called Western world. This coincides with an increase of Th2-mediated disorders such as allergic bronchial asthma and atopic dermatitis in the urban population, while these disorders are not detected in the rural population [9]. A protective effect for the development of childhood allergic diseases in rural population may be also due to the finding that Treg cells were significantly increased in farm-exposed children after phorbol 12-myristate 13-acetate/ionomycin and LPS stimulation [10]. Stimulation of innate immunity receptors can also directly influence the function of Treg cells. In this regard, it has been demonstrated that innate immunity receptors (TLRs) modulate the suppressive activity of naturally occurring CD4(+)CD25(high) Treg [11,12].

Corticosteroids are the most effective anti-inflammatory agents and topical corticosteroids including budesonide are the recommended therapy by current guidelines for asthma treatment [13]. Corticosteroids act on Tregs increasing IL-10 production [14] and enhancing IL-10 Treg function [5]. Corticosteroids inhibit T-cell activation and production of Th2 cytokines [15] and increase T cell apoptosis [16]. Corticosteroids are able to increase in vivo TLR4 expression in peripheral monocytes of asthmatics [17]. Furthermore budesonide (BUD) increases Foxp3 and IL-10 on CD4+CD25+ cells from asthmatics [18]. No study assesses the effect of corticosteroids on the expression of TLR4 and of TLR2 and IL-10 in CD4+CD25highly+ cells and the contextual release of IL-6 and of TNF-α by peripheral blood mononuclear cells (PBMCs) in asthmatics.

The objectives of this study were to assess the effects of BUD, a potent inhaled corticosteroid, on the expression of TLR4, TLR2, and IL-10 in CD4+CD25high+ lymphocytes (Treg) obtained from asthmatic patients as well as in IL-6 and TNF-α release by PBMCs.

2. Materials and methods

2.1. Subjects and study design

The study was approached by *in vitro* assessments of BUD effects in asthma. For *in vitro* evaluations, we selected 14 atopic patients with mild intermittent asthma, according to the criteria of the American Thoracic Society [19], and 11 control subjects without allergic diseases or asthma (Table 1). All asthmatic patients (Table 1) were characterized by a reversible airway obstruction assessed by an increase of ≥12% of forced expiratory volume in 1 s (FEV_1) after inhalation of 200 μg of salbutamol. The asthma diagnosis and the assessment of its severity were performed according to the Global Initiative for Asthma [20]. All recruited subjects were never-smokers. Atopy was established by aeroallergen skin prick test (Alk Abellò, Hørsholm, Denmark). The following allergens were tested by skin prick test: dermatophagoydes pteronyssinus, dermatophagoydes farinae, olive, parietaria judaica, cypress, ragweed, mugwort (Artemisia Vulgaris), cat, dog, alternaria, claudosporium. None of the patients recruited for evaluating *in vitro* effects of BUD received any corticosteroid treatment. Subjects who had bronchial or respiratory tract infections during the month before the test were not included. The study fulfilled the criteria of the Ethics Committee of Policlinico-Giaccone Hospital-Palermo, was approved (authorisation reference number 7/2013) and was in agreement with the Helsinki Declaration. All subjects had given their written informed consent.

Table 1
Demographic and clinical characteristics of the study population.

	Controls ($n = 11$)	Mild intermittent asthmatics ($n = 14$)
Caucasic race	11	14
Gender (M/F)	5/6	7/7
Age (years) 50 (25–75) percentiles	37 (26–40)	29 (23–38)
FEV1% of predicted percentiles	110 (107–119)	97 (92–105)
Bronchodilation test: Reversibility (%) percentiles	–	15 (13–15.7)
Skin prick test (negative/positive)	11/0	0/14

FEV1 = forced expiratory volume in 1 s.

2.2. Peripheral blood mononuclear cells (PBMCs) cultures

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples (10 ml) of enrolled subjects by Ficoll-Hypaque (Pharmacia) gradient centrifugation. Cells were suspended in RPMI 1640 tissue culture medium (Invitrogen Life Technologies) supplemented with 1% heat-inactivated FCS (Invitrogen Life Technologies), 2 mM L-glutamine, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-ME and 85 μg/ml gentamicin. Viability was tested using trypan blue exclusion.

For assessing *in vitro* effects of BUD, cells (2×10^6 cells/ml) were incubated in culture tubes (Becton Dickinson, Mountain View, CA) for 24 h either in the absence and in the presence of BUD (B7777 Sigma, St Louis, MO) (10^{-8} M final concentration). The concentration range of BUD 10^{-8} M and incubation time 24 h were selected on the basis of the results of a previous study [18]. In some experiments cells were cultured for 24 h with LPT (Sigma) (100 μg/ml for 18 h) and with LPS (Sigma) (1 μg/ml for 18 h) alone and combined with BUD.

2.3. Flow cytometry analysis for surface markers

PBMCs were stained with FITC anti-human CD4, PE-Cy5 anti-human CD25 and PE anti-human TLR4 or TLR2 mAbs by using FACS analysis (Becton Dickinson FACSCalibur System). All monoclonal antibodies (mAb) were from Becton Dickinson PharMingen eBioscence. Negative controls were performed using an isotype control antibody at the same titer as the primary antibodies (Becton Dickinson PharMingen). Lymphocytes were gated by forward and side scatter as previously described [18] and analysis was done on 100,000 acquired events for each sample using CellQuest acquisition and data analysis software (Becton Dickinson). Cell debris and dead cells were excluded from the analysis by forward and side scatter. The expression of TLR4 and of TLR2 was performed on total PBMC (no gating) or on lymphocyte subpopulations (CD4+CD25− and CD4+CD25+). The gate for lymphocytes was selected as previously described in a paper from our group [18]. In the experiments evaluating the effect of BUD, the expression of TLR2 and of TLR4 was assessed also in CD4+CD25highly+ cells (positivity > 10^2).

2.4. Intracellular cytokine staining

For the detection of intracellular cytokine IL-10, PBMC were cultured overnight (10 h) with GolgiStop (2 μM final concentration) (BD PharMingen). The cells were stained with mAbs against CD4 FITC and CD25 PE-Cy5 in PBS containing 1% FCS and 0.1% Na azide for 30 min at 4 °C. Cells were washed twice in PBS with 1% FCS and fixed with PBS containing 4% paraformaldehyde for 20 min at room temperature. After two washes in permeabilization buffer (PBS

containing 1% FCS, 0.3% saponin, and 0.1% Na azide) for 15 min at 4 °C, the cells were stained with 0.25 μg of PE anti-human IL-10 antibody (BD PharMingen). After two more washes in PBS containing 1% FCS, the cells were analysed by flow cytometry. The expression of IL-10 was assessed in CD4+CD25highly+ cells.

2.5. Intracellular Foxp3 staining

PBMC were simultaneously stained with PE anti-human CD4 and PE-Cy5 anti-human CD25 (BD PharMingen). Cells were fixed and permeabilized using the BD PharMingen human Foxp3 Buffer Set, following the manufacturer's recommended assay procedure. Finally, the cells were stained with FITC anti-human Foxp3 (BD PharMingen).

2.6. Multi-analyte ELISArray

Cells (2×10^6/ml) were incubated in culture tubes (Becton Dickinson, Mountain View, CA) for 24 h either in the absence and in the presence of BUD (10^{-8} M final concentration) and cell supernatants were collected. For the simultaneous detection of 12 cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17A, IFNγ, TNF-α, G-CSF, TGFβ1), in cell supernatants, a multi-analyte ELISArray TH1/TH2/TH17 (Qiagen, Valencia, CA), using a standard sandwich ELISA protocol, was performed following manufacturer's instructions. The expression of the each cytokine was expressed as absorbance. Absorbance values were corrected for dilution of the samples and negative control absorbance was subtracted. Cytokines with a baseline absorbance less than 0.1 (IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IFNγ, G-CSF) were excluded.

2.7. Statistical analysis

Data are expressed as medians and 25–75 percentiles or as mean ± SD. All the statistical analyses were performed using the StatView 5.0.1 software. A non-parametric Mann Whitney test for comparisons between the two recruited groups was applied. Statistical analysis of the *in vitro* effects of BUD was performed by Wilcoxon test or by paired t test. Kolmorogov–Smirnov Normality test was initially performed to assess whether parametric analyses of data could be performed. $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. Demographic characteristics of the subjects

The demographic characteristics, the clinical and functional evaluations of the studied patients are shown in Table 1.

3.2. TLR2 and TLR4 expression by PBMCs

Initially we tested whether a different expression of innate immunity receptors, TLR2 and TLR4, was present on PBMCs from controls and asthmatics. TLR2 (Fig. 1) and TLR4 (Fig. 2) expression was significantly reduced in asthmatics in comparison to controls.

3.3. TLR2 and TLR4 expression by lymphocyte subpopulations

We next assessed the distribution of TLR2 and TLR4 expression in CD4+CD25+ and in CD4+CD25− lymphocyte subpopulations. As shown in Fig. 3A–B, CD4+CD25+ cells highly expressed TLR2 and TLR4 molecules in comparison to CD4+CD25− cells in both controls and in asthmatics. Representative dot plots are shown in Figure S1. CD4+CD25+ cells highly expressed Foxp3 molecule in comparison to CD4+CD25− cells in both controls and in asthmatics confirming that Treg cells are mainly present on CD4+CD25+ cells (Fig. 4). Furthermore, the expression of Foxp3 on CD4+CD25+ cells was significantly lower in asthmatics than in controls ($p < 0.03$; Mann Whitney test). Among the CD4+CD25+ cells, the higher percentage of Foxp3+ cells was present in CD4+CD25highly+ cells (CD25 low+ 6.2 ± 2.2; CD25highly+ 14.2 ± 4.5).

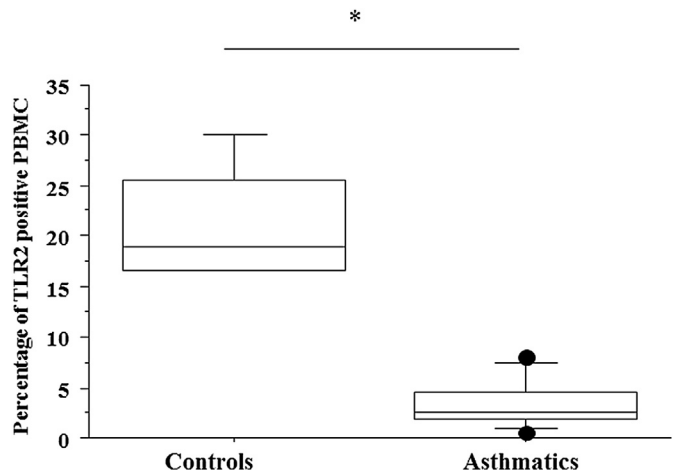

Fig. 1. Expression of TLR2 in PBMC. PBMC from controls ($n = 8$) and from mild intermittent asthmatics ($n = 11$) were isolated and were assessed for TLR2 expression by flow-cytometry. Data are expressed as percentage of positive cells. Horizontal bars inside boxes represent the median values and limits of boxes represent the 25th and 75th percentiles. $^*p < 0.05$ Mann Whitney test for comparisons between controls and asthmatics.

3.4. Effect of BUD on TLR2 and TLR4 expression by CD4+CD25highly+ cells

Since the expression of TLR in Treg may regulate the activity of these cells [10], the effect of BUD on the expression of both TLR2 and TLR4 in CD4+CD25highly+ cells from asthmatics was also explored.

BUD was able to significantly increase the expression of TLR2 (Fig. 5) and of TLR4 (Fig. 6) in CD4+CD25highly+ cells. BUD was not

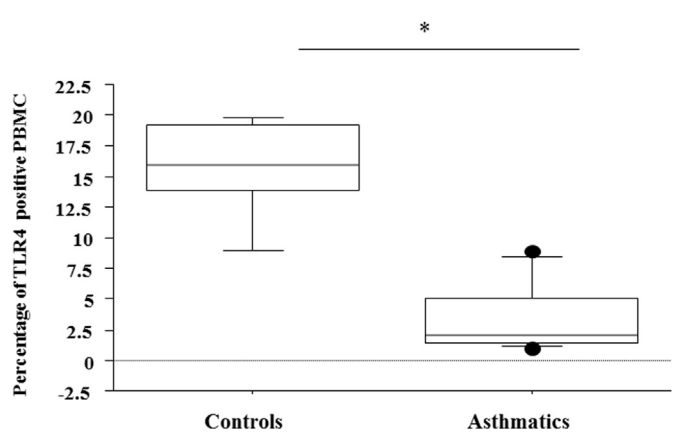

Fig. 2. Expression of TLR4 in PBMC. PBMC from controls ($n = 8$) and from mild intermittent asthmatics ($n = 11$) were isolated and were assessed for TLR4 expression by flow-cytometry. Data are expressed as percentage of positive cells. Horizontal bars inside boxes represent the median values and limits of boxes represent the 25th and 75th percentiles. $^*p < 0.05$ Mann Whitney test for comparisons between controls and asthmatics.

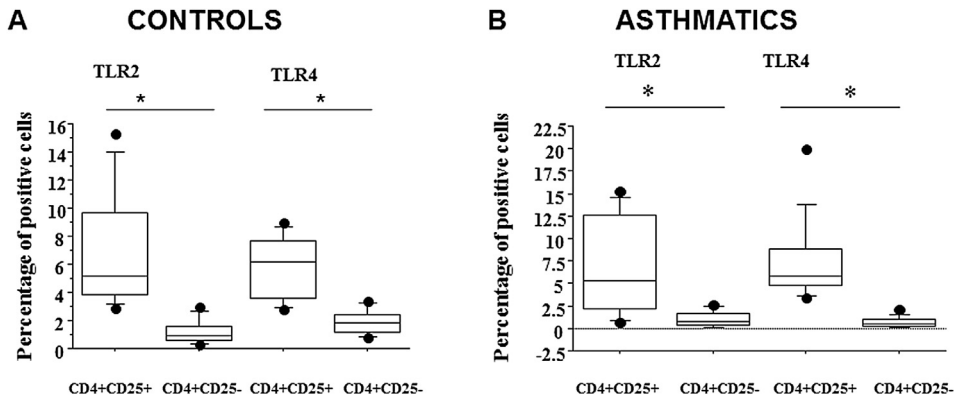

Fig. 3. Expression of TLR2 and TLR4 in lymphocyte subpopulations. PBMC from controls ($n = 8$) and from mild intermittent asthmatics ($n = 11$) were isolated and the total lymphocytes were gated by forward and side scatter. The expression of TLR4 and of TLR2 was assessed on lymphocyte subpopulations (CD4+CD25− and CD4+CD25+). Data are expressed as percentage of positive TLR2 and TLR4 in lymphocyte subpopulations of controls (C) and of asthmatics (D). Horizontal bars inside boxes represent the median values and limits of boxes represent the 25th and 75th percentiles. $^*p < 0.05$ Wilcoxon test for comparing TLR2 and TLR4 expression in CD4+CD25+ and in CD4+CD25− cells within controls and asthmatics.

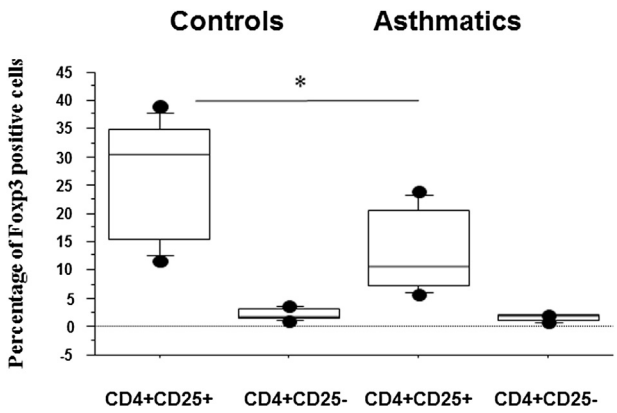

Fig. 4. Expression of Foxp3 in lymphocyte subpopulations. PBMC from controls ($n = 8$) and from mild intermittent asthmatics ($n = 11$) were isolated and the total lymphocytes were gated by forward and side scatter. The expression of Foxp3 was assessed on lymphocyte subpopulations (CD4+CD25− and CD4+CD25+). Data are expressed as percentage of Foxp3 in lymphocyte subpopulations of controls and of asthmatics. Horizontal bars inside boxes represent the median values and limits of boxes represent the 25th and 75th percentiles. $^*p < 0.03$; Mann Whitney test comparing the expression of Foxp3 in CD4+CD25+ cell population between controls and asthmatics.

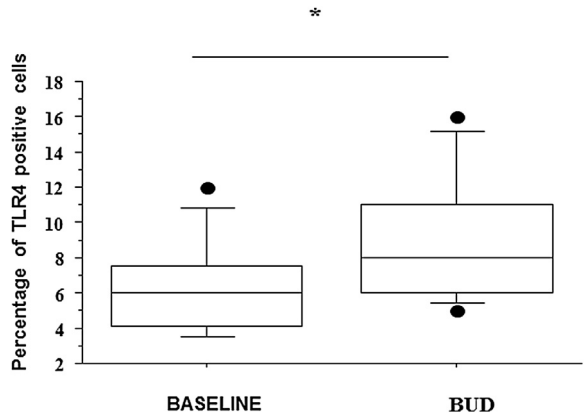

Fig. 6. Effect of BUD on the expression of TLR4 on CD4+CD25highly+ cells from asthmatics. PBMC from mild intermittent asthmatics ($n = 11$) were cultured with/without BUD and were assessed for TLR4 expression by flow-cytometry in CD4+CD25highly+. Data are expressed as percentage of positive cells. Horizontal bars inside boxes represent the median values and limits of boxes represent the 25th and 75th percentiles. $^*p < 0.05$ Wilcoxon test.

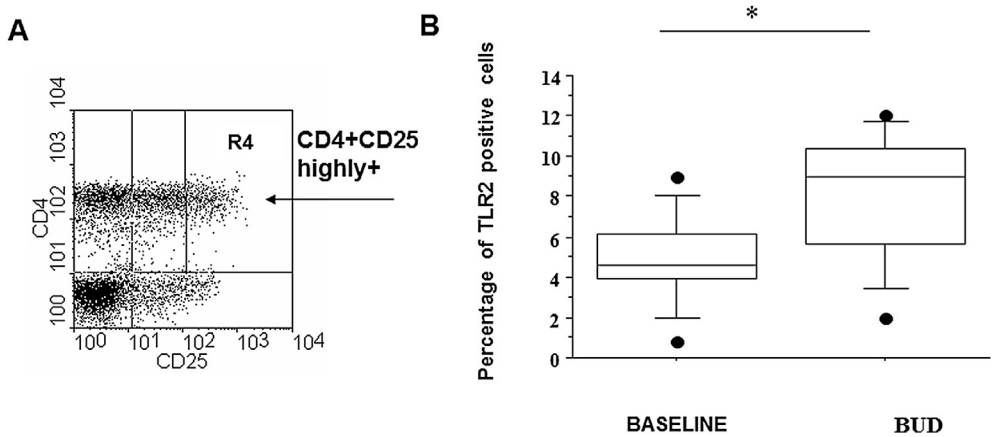

Fig. 5. Effect of BUD on the expression of TLR2 in CD4+CD25highly+ cells from asthmatics. PBMC from mild intermittent asthmatics ($n = 11$) were cultured with/without BUD and were assessed for TLR2 expression by flow-cytometry in CD4+CD25highly+ cells. CD4+CD25highly+ cells were gated as shown in A. Data are expressed as percentage of positive cells (B). Horizontal bars inside boxes represent the median values and limits of boxes represent the 25th and 75th percentiles. $^*p < 0.05$ Wilcoxon test.

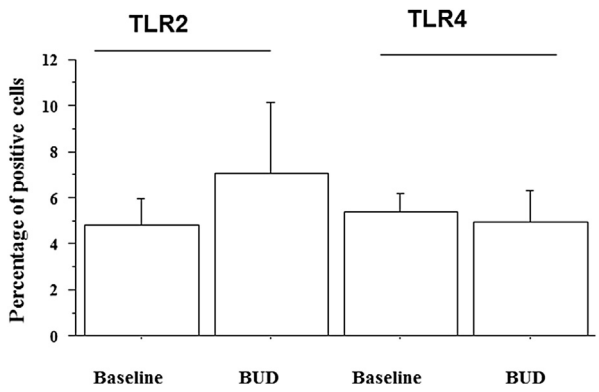

Fig. 7. Effect of BUD on the expression of TLR2 and of TLR4 on lymphocytes from controls. PBMC from controls ($n = 3$) were cultured with/without BUD and were assessed for TLR2 and TLR4 expression by flow-cytometry. Data are expressed as percentage of positive cells. Horizontal bars inside boxes represent the median values and limits of boxes represent the 25th and 75th percentiles. $^*p < 0.05$ Wilcoxon test.

able to significantly modify TLR2 and TLR4 expression in CD4+CD25+ lymphocytes from controls (Fig. 7).

3.5. Effect of TLR2 and TLR4 ligands on Foxp3 expression in lymphocytes

Foxp3 is a transcription factor specifically associated to regulatory activities. We next tested whether a TLR2 ligand, LPT and a TLR4 ligand (LPS) induced Foxp3 expression on lymphocytes from both controls and asthmatics. LPS was able to significantly induce Foxp3 expression in total lymphocytes and in CD4+CD25− lymphocytes from asthmatics. LPS was also able to significantly induce Foxp3 expression in CD4+CD25− lymphocytes from controls. No significant effect was observed for LPT (Fig. 8).The combined presence of TLR2 and TLR4 ligands and BUD did not increase Foxp3 expression (data not shown).

3.6. Effect of BUD on IL-10 expression by CD4+CD25high+ cells

Corticosteroids act on CD4+CD25+ cells from asthmatics increasing IL-10 production [12,18]. In CD4+/CD25highly+ cells from asthmatic patients BUD was able to increase the percentage of IL-10 positive cells (Fig. 9).

3.7. Effect of BUD on IL-6 and TNF-α release by PBMCs from asthmatics

The simultaneous detection of 12 different Th1/Th2/Th17 cytokines was performed. Among the analysed cytokines only IL-6 and TNF-α were evaluable (absorbance values more than 0.1) at baseline and were modulated by BUD. When PBMCs from asthmatics were incubated with BUD a significantly reduced release of IL-6 (Fig. 10) and of TNF-α (Fig. 11) was observed. IL-17 and TGFβ1 had absorbance values at the baseline more than 0.1 but were not significantly modulated by BUD (data not shown).

4. Discussion

Allergic diseases including bronchial asthma are not confined to the area where they originated but they involve also systemic circulation [21]. The present study demonstrates that BUD, an inhaled corticosteroid widely used for the management of bronchial asthma, controls asthma inflammation by modulating TLR2 and TLR4 and IL-10 expression in CD4+/CD25highly+ cells as well as IL-6 and TNF-a release in PBMC of asthmatics. These mechanisms of action of BUD provide further understanding on new mechanisms by which corticosteroids control T lymphocyte activation, an important target of anti-inflammatory drugs.

The hygiene hypothesis is considered the most reasonable explanation for the allergy epidemics in Western countries that has occurred over the past few decades [8]. The immunological mechanisms on the basis of the hygiene hypothesis are a missing immune deviation of allergen specific responses from a Th2 to a Th1 profile and a reduced activity of Treg cells [10]. Innate immunity receptors (TLRs) may directly modulate the suppressive activity of naturally occurring CD4(+)CD25(highly+) Treg [11,12]. Accordingly, Treg cells are increased in farm-exposed children after LPS stimulation, the agonist of TLR4 [10]. Furthermore, TLR4 signalling may promote the induction of IL-10-secreting type 1 regulatory T cells [22] and a reduced activation [23] or expression [24] of specific TLRs may account for a missing immune deviation of allergen specific responses from a Th2 to a Th1 profile. In this regard, a previous study demonstrates that an up-regulation of TLR2 and TLR4 expression in PBMCs from asthmatics is associated to reduced pro-inflammatory responses to allergens [25]. On the basis of these concepts, we initially evaluated the expression of TLR2 and TLR4 and demonstrated a reduced expression of these receptors in PBMCs, i.e. total lymphocytes and monocytes from untreated asthmatics in comparison to controls. Since the expression of TLR2

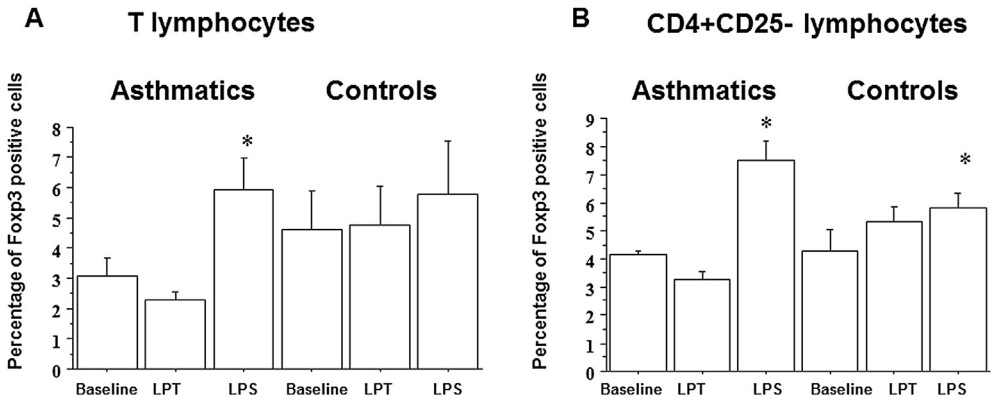

Fig. 8. Effect of TLR2 and TLR4 ligands on the expression of Foxp3 on lymphocytes from controls and from asthmatics. PBMC from controls ($n = 3$) and from asthmatics ($n = 3$) were cultured with/without a TLR2 ligand (LPT) and a TLR4 ligand (LPS) and were assessed for Foxp3 expression by flow-cytometry. A. Foxp3 expression on total lymphocytes. B. Foxp3 expression on CD4+CD25− lymphocytes. Data are expressed as percentage of positive cells (mean ± SD). $^*p < 0.05$ paired t test.

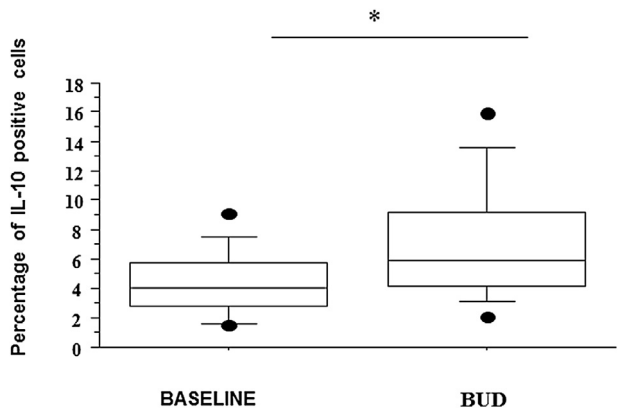

Fig. 9. Effect of BUD on the expression of IL-10 in CD4+CD25highly+ cells from asthmatics. PBMC from mild intermittent asthmatics ($n = 11$) were cultured with/without BUD and were assessed for IL-10 expression by flow-cytometry in CD4+CD25highly+ cells. Data are expressed as percentage of positive cells. Horizontal bars inside boxes represent the median values and limits of boxes represent the 25th and 75th percentiles. $^*p < 0.05$ Wilcoxon test.

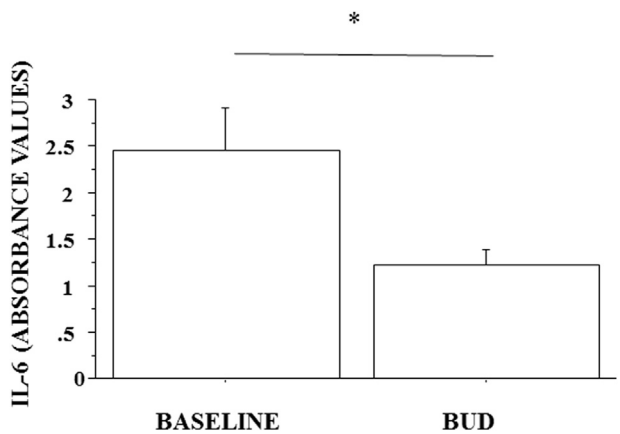

Fig. 10. Effect of BUD on IL-6 release by PBMC from asthmatics. PBMC from mild intermittent asthmatics ($n = 3$) were cultured with/without BUD and IL-6 release was assessed by multi-analyte ELISArray. Data are expressed as absorbance (mean ± SD). $^*p < 0.05$ paired t test.

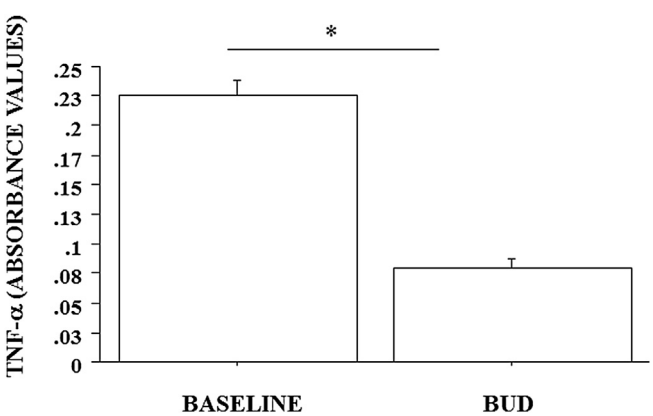

Fig. 11. Effect of BUD on TNF-α release by PBMC from asthmatics. PBMC from mild intermittent asthmatics ($n = 3$) were cultured with/without BUD and TNF-α release was assessed by multi-analyte ELISArray. Data are expressed as absorbance (mean ± SD). $^*p < 0.02$ paired t test.

and TLR4 in both controls and asthmatics were mainly concentrated in CD4+CD25+ cells, we further assessed whether BUD affected the expression of TLR2 and TLR4 innate immune receptors in a subpopulation of CD4+CD25+ cells, i.e. on CD4+CD25highly+ cells, the naturally occurring Treg [11,12]. We demonstrated that BUD increases the expression of both TLR2 and TLR4 in CD4+CD25highly+ from recruited asthmatics but not from recruited controls. Consequently, BUD via the up-regulation of innate immune receptors on Treg may have the potential to ameliorate the Th2 immune deviation of allergic subjects. To further demonstrate this effect, we stimulated lymphocytes from asthmatics and controls with TLR2 and TLR4 ligands. It was demonstrated here that LPS, a TLR4 ligand is able to increase Foxp3 in CD4+CD25− in asthmatics as well as in controls. This phenomenon may be relevant in the control of bronchial inflammation since CD4+/CD25− T lymphocytes ectopically expressing Foxp3 acquire the function to control inflammation in experimentally induced inflammatory bowel disease and to down-regulate the proliferation of CD4+/CD25− T lymphocytes *in vitro* [26].

Although it has been previously demonstrated by our group that BUD *in vitro* is able to significantly increase the percentage of Foxp3 positive cells in CD4+CD25− [18], the combined presence of TLR2 and TLR4 ligands and BUD did not induce any effects on Foxp3 expression. Further experiments are needed to clarify this phenomenon.

T regulatory activities are mainly related to naturally occurring CD4+/CD25+ Tregs and to CD4+/CD25− inducible Tregs [27]. It has been previously demonstrated that BUD increases costimulatory activities in CD4+/CD25+ further supporting the concept that this drug is effective in controlling T lymphocyte activation also improving the function and the activities of the Tregs. Here, we confirm that Foxp3, a transcription factor typically associated to Tregs activities, is expressed on CD4+/CD25+ [28] and more on CD4+CD25highly+. This natural CD4+/CD25+ subset is thymus-born, constitutively expresses IL-10 mRNA, does not produce IL-2, and is resistant to apoptosis [28].

T regulatory activities have the potential to suppress pathogenic Th2 responses thus preserving lung integrity [28] and may be defective or overridden in patients with allergic diseases including asthma [5]. The levels of Foxp3 mRNA in BAL from children with asthma are lower than in healthy controls, positively correlate with FEV1 and, after 4 weeks of treatment with inhaled corticosteroids, significantly increase [3]. Furthermore, in severe asthmatics, the Foxp3 protein expression in PBMC correlates with the FEV1 values and with the symptom score [29]. Here we confirm as previously reported [30] that although the total numbers of CD4+CD25highly+ cells in controls and in asthmatics were similar (data not shown), CD4+CD25+ cells from asthmatics have lower expression of Foxp3 in comparison to controls. As previously reported [18], not significant differences were observed for the numbers of CD4+CD25+ cells between asthmatics and controls.

BUD *in vitro* is able to significantly increase Treg activities and to down-regulate the lymphocyte proliferation upon allergen stimulation [18,31]. Tregs control effector immune responses through a diverse array of mechanisms including secretion of the anti-inflammatory cytokines TGF and IL-10 [32]. IL-10 is essential not only for suppression of effector cells by Treg cells but also for their differentiation [27]. IL-10, produced by a number of different cells, affecting antigen presenting cell function, dendritic cell maturation as well as the activation of co-stimulatory molecules, can inhibit both Th1 and Th2 type responses [14]. IL-10 activates tyrosin phosphatase 1 which dephosphorylates rapidly CD28 and ICOS co-stimulatory receptors [33]. We see here that BUD is able to increase the expression of IL-10 in CD4+/CD25+ cells. A rapid switch and expansion of IL-10-producing cells and the use of multiple

suppressive factors represent essential mechanisms in immune tolerance to a high dose of allergens in non-allergic individuals [34].

Moreover, the present study demonstrates for the first time that BUD is able to significantly reduce the release of IL-6 and TNF-α by PBMCs from asthmatics. TNF-α may be released by CD4+ and CD8+ lymphocytes in peripheral blood compartment [35].

IL-6 is a small size glycoprotein (21 KDa) produced by cells from the innate immune system (e.g. macrophages, dendritic cells, mast cells, neutrophils), but also by B cells and, to a lesser extent, by some CD4 effector Th cells [36]. Increased levels of IL-6 in serum in asthmatic patients, may contribute to asthma pathogenesis, regulating the effector CD4 T cell fate, promoting IL-4 production during Th2 differentiation, inhibiting Th1 differentiation and, together with TGFβ, promoting Th17 cell differentiation and suppressing regulatory T-cells [6,37,38]. In this regard, it was found that TGFβ induces the generation of Foxp3 Treg, which were completely inhibited by a pro-inflammatory cytokine IL-6 [7]. In addition, it has been shown that the levels of IL-6 in sputum inversely correlate with FEV1 values [39].

Taken together these findings suggest that IL-6 is likely to be directly involved in the pathogenesis of asthma and in the progressive loss of lung function observed in patients who remain untreated; i.e. IL-6 is likely a potential target for new treatments for this important disease.

Moreover, IL-6 has been considered a general marker of inflammation together with TNF-α and IL-1β, two other classical inflammatory cytokines. TNF-α has been implicated in the pathogenesis of asthma, and neutralization of TNF-α is an effective therapy for inflammatory diseases [40]. In children the up-regulation of TNF-α expression is associated to a more severe course of respiratory syncytial virus infection as well as to the onset of asthma [41]. Furthermore, circulating levels of TNF-α are increased in severe asthmatics more than in mild and moderate asthmatics [42]. Unfortunately, the need to recover millions of cells to perform all the analyses to test BUD effects did not allow us to extend the study to lymphocytes isolated from induced sputum or bronchoalveolar lavages.

In conclusion, these results suggest that BUD is effective in controlling asthma inflammation up-regulating the expression of TLR2/4 and IL-10 in Treg and down-regulating the release of pro-inflammatory cytokines (IL-6 and TNF-α) with an important role in the pathogenesis of asthma. Future studies are needed to clarify whether these immune-modulatory effects of BUD are shared or not with other inhaled steroids.

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Caterina Di Sano, Andreina Bruno and Maria Ferraro performed all the experiments of the study and participated to the interpretation of the data.

Valentina Caputo and Salvatore Gallina participated to the interpretation of the data.

Mark Gjomarkaj contributed to the interpretation of the data and to the writing out of the manuscript.

Appendix A. Supplementary material

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pupt.2015.02.003.

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