



Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm



Crosstalk between T cells and bronchial fibroblasts obtained from asthmatic subjects involves CD40L/ α 5 β 1 interaction

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ARTICLE INFO

Article history:

Received 22 December 2009

Received in revised form 15 March 2010

Accepted 18 March 2010

Available online xxx

Keywords:

Asthma

Bronchial fibroblasts

T cells

CD40L

Integrins

α 5 β 1

Allergy

ABSTRACT

Background: Allergic asthma is characterized by infiltration of inflammatory cells into the airways. T cell-derived cytokines regulate both airway inflammation and remodelling. In the human airways, T cell–fibroblast interactions may have a role in regulating inflammation and remodelling.

Objectives: To evaluate the effect of bronchial fibroblast–T cell interaction on profibrogenic cytokine release and determine the nature of the molecules involved in this interaction.

Methods: Human bronchial fibroblasts obtained from healthy and asthmatic donors were co-cultured with purified T cells derived from peripheral blood of the same subjects. IL-6 mRNA and protein levels were measured by real time PCR and ELISA. CD40, CD40L and α 5 β 1 were evaluated by flow cytometry. Bronchial fibroblasts were stimulated with rsCD40L. Neutralisation was performed using neutralizing antibodies anti-CD40L and anti- α 5.

Results: Contact of T cells with bronchial fibroblasts up-regulated IL-6 at both gene and protein levels. This effect was significantly higher in fibroblasts from asthmatics than those from controls. Blocking CD40L and α 5 β 1 integrin showed a significant inhibition of IL-6 expression in asthmatics but not in healthy controls. Stimulation of fibroblasts with recombinant soluble CD40L up-regulated IL-6 production in asthmatics but not in controls. Adhesion to fibronectin, a α 5 β 1 integrin ligand, is increased in fibroblasts from asthmatics compared to fibroblasts from controls.

Conclusion: These results showed that interaction of bronchial fibroblasts with T cells increases the production of profibrogenic cytokine IL-6. In asthmatic condition this interaction involves CD40L/ α 5 β 1. These results suggest that T cells and structural cells crosstalk in asthma may maintain local mucosal inflammation.

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1. Introduction

Allergic asthma is a complex and heterogeneous disease characterized by bronchial hyperreactivity, chronic inflammation and the activation of inflammatory cells (Busse et al., 2001). T lympho-

cytes play a major role in asthma. In both lavage and in bronchial biopsies, T cells exhibit increased expression of the cell surface activation markers, which appears to correlate with clinical indices of disease activity (Wilson et al., 1992; Redington et al., 2000). These activated T cells exhibit a Th2 profile preferentially producing IL-4, IL-5, GM-CSF, and IL-13 (Robinson et al., 1993; Ying et al., 1997). Asthmatic inflammation is believed to trigger an injury-repair cycle in the bronchial wall leading to airway remodelling (Fish and Peters, 1999). Remodelling can be defined as a change in composition, content and organization of the cellular and molecular constituents of the airway wall. These changes include epithelial disorganization, basement membrane thickening, hyperplasia and hypertrophy of the bronchial smooth muscle and deposition of extracellular matrix (ECM) protein (Fish and Peters, 1999; Ward et al., 2002). The latter response is most prominent in the lamina reticularis and results from the deposition of interstitial collagen, fibronectin and proteoglycan. It has been proposed that airway structural changes

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ECM, extra cellular matrix; FBS, foetal bovine serum; FEV1, forced expiratory volume in 1 s; GM-CSF, granulocyte macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule-1; IL-4, interleukin-4; IL-5, interleukin-5; IL-6, interleukin-6; mAb, monoclonal antibody; mRNA, messenger ribonucleic acid; TGF, transforming growth factor; VCAM-1, vascular cell adhesion molecule-1; VLA-5, very late antigen-5.

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contribute to the development of chronic airway hyperresponsiveness and the progressive deterioration in lung function over time (Ward et al., 2002). Changes in the airway structure may promote continued Th2 cell activation locally.

The mucosal microenvironment composed of structural cells and highly specialised ECM is able to amplify and promote inflammation in the airways. *In vitro* and animal studies showed that interactions of inflammatory cells with structural cells play an important role in airway inflammation and remodelling (Ramos-Barbon et al., 2005; Hamilton et al., 2001; Plante et al., 2006). Structural cells, like bronchial fibroblasts, are able to produce multiple mediators that can play a role in maintaining and amplifying the inflammatory response in asthma. Recent studies have shown that fibroblasts actively define the structure of tissue microenvironments and modulate immune cell behaviour by conditioning the local cellular and cytokine microenvironment (Vancheri et al., 2005). Thus, fibroblasts have been shown to modulate leukocyte survival and to regulate their differentiation (Parsonage et al., 2005). Fibroblasts express many surface adhesion and co-stimulatory molecules related to leukocyte trafficking and signalling such as ICAM-1, VCAM-1 (Bombara et al., 1993) as well as CD40 (van Kooten and Banchereau, 2000). Indeed, ICAM-1/LFA-1 and CD40/CD40 ligand (CD40L) were shown to mediate leukocyte recruitment and the development of the IgE response in the airways (Mehlhof et al., 2000; Keramidaris et al., 2001). Recently, Leveille et al. showed that CD40L can interact with $\alpha 5 \beta 1$ (VLA-5) and triggers cell signalling leading to cytokine expression in U937 cells (Leveille et al., 2007). $\alpha 5 \beta 1$ is a member of the integrins, an ubiquitously expressed and highly-conserved family of adhesion receptors involved in cell-ECM, as well as cell-cell adhesion (Leveille et al., 2007; Hynes, 1999). $\alpha 5 \beta 1$ has been identified as the key receptor for fibronectin in different cells (Yasuda et al., 1995). Fibronectin is increased in asthmatic airways (Chakir et al., 1996). Various mediators, including interleukins (Seminario and Bochner, 1997) and chemokines (Lloyd et al., 1996), can modulate $\alpha 5 \beta 1$ adhesion. Fibroblasts have been shown to produce a large array of cytokines, such as IL-6 (Smith et al., 1997), which is considered a multifunctional cytokine. IL-6 levels are increased in blood, bronchoalveolar lavage fluid (BALF), and the lung tissues of asthmatic patients (Doganci et al., 2005). Moreover, IL-6 derived from antigen-presenting cells is able to induce initial IL-4 production in naive CD4⁺ T cells, thereby polarizing these cells into Th2 cells (Rincon et al., 1997). Targeted over-expression of IL-6 in transgenic mice lungs leads to the development of sub-epithelial fibrosis (Kuhn et al., 2000). Fibrosis is also reduced in IL-6 knockout mice (Natsume et al., 1999). Furthermore, in tissues such as the liver, skin, and lungs, IL-6 expression has been shown to correlate with fibrosis (Kayano and Okita, 2000; Sato et al., 2001; Smith et al., 1998). In this study, we investigated the role of cell-cell interactions between bronchial fibroblasts and T lymphocytes in the production of IL-6 by bronchial fibroblasts in asthma and the implication of the CD40L/ $\alpha 5 \beta 1$ integrin in this process.

2. Methods

2.1. Subjects

Asthmatic subjects fulfilling the American Thoracic Society criteria for asthma (Gardner and Hankinson, 1988) were recruited from the Asthma Clinic at the Institut Universitaire de Pneumologie et de Cardiologie de Québec. All asthmatic subjects (mean age, 23 years; mean FEV₁, 97.4 ± 2.6%; mean PC₂₀, 2.5 ± 1.1 mg/mL) were atopic with at least one positive response to common allergens on allergy skin prick tests. They used only an inhaled β_2 -agonist on demand and did not use inhaled or systemic corticosteroids. In

the month preceding the study, none of the subjects reported a respiratory infection or an increase in asthma symptoms. Healthy subjects (mean age, 26 years; mean FEV₁, 101.7 ± 3.6%; mean PC₂₀, 83.8 ± 16.1 mg/mL) were non-atopic non-smokers with no history of asthma or atopy. The study was approved by our local Ethics Committee and following receipt of the subjects' informed written consent.

2.2. Bronchial fibroblast isolation and culture

Fibroblasts were isolated from bronchial biopsies as described previously (Chakir et al., 2001). Cultured fibroblasts were identified and characterized by immunofluorescence and flow cytometry using anti-vimentin and a mouse anti-human fibroblast antigen Ab-1 antibody (Calbiochem, San Diego, CA, USA). The purity of fibroblast cell culture was 98% (Chakir et al., 1996). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/mL penicillin/streptomycin (Gibco, Burlington, ON, Canada) and 10% FBS (Gibco). The media was changed three times a week. Cells were used at early passages to keep their phenotype (Dube et al., 1998).

2.3. T lymphocyte isolation

Healthy and asthmatic subjects, from whom structural bronchial fibroblast cells were derived, donated blood which was used as a source of T lymphocytes. Mononuclear cells were isolated from peripheral blood using Ficoll-Paque gradient (GE Healthcare, Buckinghamshire, UK) by centrifugation at 700 × g for 20 min. Cells were washed with RPMI containing 10% of heat-inactivated FBS culture medium and incubated in a culture flask to allow monocytes to adhere. Non-adhering cell suspensions were then purified on a Nylon column (Polysciences Inc., Warrington, PA, USA). To assess the purity, cells were stained with anti-CD3 and anti-CD19 (BD Bioscience, San Diego, CA, USA) and analyzed by flow cytometry that revealed 95% of the purified cells were CD3⁺ T cells.

2.4. Fibroblast and T lymphocyte co-cultures

Healthy and asthmatic bronchial fibroblasts were plated at 5 × 10⁵ cells per well in a 6-well culture plates in 10% FBS-supplemented DMEM and allowed to adhere overnight at 37 °C in a humidified incubator and 5% CO₂ atmosphere. The following day, freshly isolated T lymphocytes (5 × 10⁶) were added to bronchial fibroblasts. The T lymphocytes were co-cultured with the fibroblasts for 6–24 h. Supernatants were harvested and frozen and the T lymphocytes were collected by extensive washing. The fibroblasts were then detached by trypsin and the presence of contaminating T cells was evaluated by flow cytometry using anti-CD18. The purity of the retrieved fibroblasts was 99%. The RNA was extracted from each cell population or cells was analyzed by flow cytometry.

2.5. Real time PCR analysis

Total RNA was extracted from both cell types using the RNeasy kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. Concentrations of total RNA were measured by a fluorescent dye with the Ribogreen RNA quantification reagent (Molecular Probes Inc., Eugene, OR, USA). 1 µg of total RNA per sample were denatured at 65 °C for 10 min and incubated at 37 °C for 60 min, with the following mix: 0.5 mM dNTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 0.25 µg/ml hexamer (Amersham Pharmacia Biotech), 200 U RT-MMLV and 1 × first strand buffer (Invitrogen). IL-6 and GAPDH were amplified by qPCR using the SybrGreen reagent (Qiagen) on an Opticon-2 system (MJ Research Inc., Waltham, MA, USA). IL-6 was amplified with the

following primers: 5'-TCT CCA CAA GCG CCT TCG-3' as sense and 5'-CTG AGG GCT GAG ATG CCG-3' as antisense. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer sequences were: 5'-ATG CAA CGG ATT TGG TCG TAT-3' as sense and 5'-CTG AGG GCT GAG ATG CCG-3' as antisense. PCR products amplified were at 150 and 221 bp, respectively. Real Time PCR were migrated onto agarose gel and compared to a standard DNA ladder for validation.

2.6. ELISA

Multiple aliquots of co-culture supernatants were collected and frozen at -80°C . IL-6 production was measured using a commercially available ELISA kit according to the manufacturer's instructions (R&D Systems).

2.7. Flow cytometry analysis

Fibroblasts were detached from culture plates prior to staining by incubation with 0.025% trypsin for 3 min at room temperature. Cells were stained with mouse anti-human antibodies specific for anti- $\alpha 5\beta 1$ mAb hybridoma HA5 (Chemicon, Billerica, MA, USA), anti-CD40 mAb hybridoma G28.5 (mouse IgG1), or with mouse IgG control (BD Pharmingen). T cells were stained with anti-CD40L hybridoma 5C8 (a gift from Dr. Mourad) diluted in a PBS/0.05% saponin (Sigma–Aldrich) or mouse IgG control (BD Pharmingen, ON, Canada). In each case, a secondary goat anti-mouse FITC conjugated antibody (BD Pharmingen) was used. Analyses were performed with an EPICS[®] ELITE ESP flow cytometer (Beckman-Coulter, Miami, FL, USA).

2.8. Neutralization studies

Prior to co-culture, T cells (5×10^6) were pre-incubated for 1 h at 37°C with anti-CD40L mAb hybridoma 5C8. In another set of experiments, fibroblasts were pre-incubated for 1 h at 37°C with anti-CD40 mAb hybridoma G28.5 ($5 \mu\text{g/mL}$), with anti-integrin $\alpha 5$ mAb hybridoma P1D6 ($10 \mu\text{g/mL}$, Chemicon), or with the isotype-matched control antibodies. Cells were washed, co-cultured for 6 h and used for mRNA analysis.

2.9. Stimulation of fibroblasts with recombinant soluble CD40L

Bronchial fibroblasts were stimulated with different concentrations (1, 2 and $5 \mu\text{g/mL}$) of recombinant trimeric soluble CD40L (rsCD40L) + $2 \mu\text{g/mL}$ of ligand enhancer, both from Alexis Biochemical (San Diego, CA, USA) for 3 h. Cells were then washed and used for total RNA extraction.

2.10. Binding assay

Bronchial fibroblasts were incubated for 1 h at 37°C with rsCD40L (1, 2, and $5 \mu\text{g/mL}$, Alexis Biochemical, San Diego, CA, USA) and with $10 \mu\text{g/mL}$ of anti-integrin $\alpha 5$ mAb (Chemicon), prior to being stimulated with rsCD40L (Alexis Biochemical). The cells were washed and blocked with PBS/2% rat serum prior to incubation with a FITC conjugated anti-human CD40L (BD Pharmingen) for 1 h on ice, then were re-washed and subjected to flow cytometry analysis.

2.11. Cell adhesion assay

The adhesion test was performed as previously described (Boisvert et al., 2007). Briefly, fibronectin was coated on a 96-well plate for 24 h at 4°C . The following day, bovine serum albumin (BSA) 1% was added for 30 min at 37°C to inhibit unspecific attachment. 5×10^4 bronchial fibroblasts were previously incubated or not with anti-integrin $\alpha 5$ mAb ($10 \mu\text{g/mL}$, Chemicon) for 1 h,

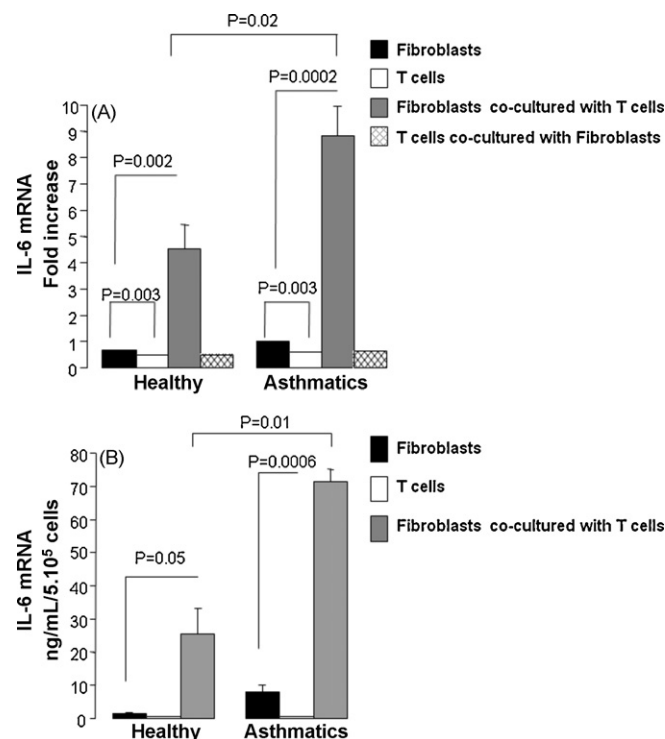


Fig. 1. T cells stimulate IL-6 production by bronchial fibroblasts. (A) Quantitative PCR analysis of IL-6 mRNA. T cells and fibroblasts from the same subjects were co-cultured for 6 h and then T cells were removed by PBS washing. RNA was then extracted from both the T cells and the fibroblasts. Data are respectively presented as the fold increase over fibroblast IL-6 mRNA expression in the absence of T cells for fibroblasts, and as fold induction over T cell IL-6 mRNA expression in the absence of fibroblasts for T cells. (B) IL-6 was measured in culture supernatants after 24 h of co-culture. Data are presented as mean $\pm$ SEM of 8 asthmatics and 8 healthy controls.

after which, the fibroblasts were added to the fibronectin layer and allowed to adhere for 1 and 2 h at 37°C . Cells were washed with PBS, fixed in 1% paraformaldehyde at room temperature for 1 h and then supplemented with 0.5% violet crystal for 1 h. After 5 washes, cells were lysed with SDS 1% for 5 min and the absorbance was measured at 580 nm with an ELISA plate reader.

2.12. Statistical analysis

All of the experiments in this study were performed at least three times. We used an SAS software package and a statistician performed an analysis. *A posteriori*, multiple comparisons were performed using the Tukey–Kramer test. Mean values $\pm$ SEM of quantitative variables were used as representative measures. All reported *p* values were two-sided and were declared significant at 0.05 levels.

3. Results

3.1. Co-culture of T cells and bronchial fibroblasts

The co-culture of bronchial fibroblasts in direct contact with the T cells induced IL-6 gene expression by bronchial fibroblasts. This contact-mediated increase in IL-6 expression was significantly higher in the fibroblasts from the asthmatics (AF) (8.8 ± 1.1 -fold increase; $p=0.0002$) than in the fibroblasts from the healthy controls (HF) (4.2 ± 0.9 -fold increase; $p=0.002$). There was no significant change in IL-6 gene expression by T cells following co-culture with fibroblasts (Fig. 1A). IL-6 up-regulation was also observed at protein level ($1.4 \pm 0.3 \text{ ng/mL/} 5 \times 10^5 \text{ cells}$ vs $25.4 \pm 7.8 \text{ ng/mL/} 5 \times 10^5 \text{ cells}$; $p=0.05$ in HF and

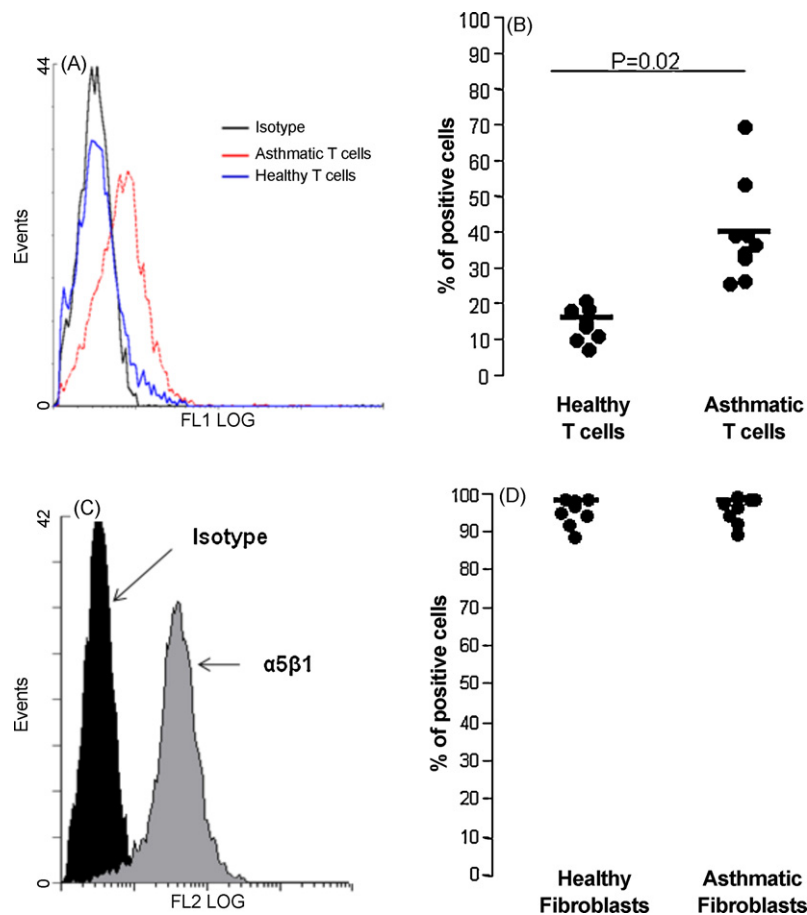


Fig. 2. Flow cytometry expression of CD40L and $\alpha 5\beta 1$. (A and B) Representative flow cytometry results and percentages of CD40L-positive T cells, respectively. (C and D) Representative flow cytometry results and dot plot of $\alpha 5\beta 1$ expression on fibroblasts. Results are representative of nine individual experiments.

7.8 ± 2.2 ng/mL/ 5×10^5 cells vs 71.4 ± 3.7 ng/mL/ 5×10^5 cells; $p = 0.0006$ in AF) (see Fig. 1B).

3.2. CD40L, CD40 and $\alpha 5\beta 1$ expression

To examine the role of CD40L/ $\alpha 5\beta 1$ integrin interaction in IL-6 production, we first determined the CD40L expression level on the T cells and that of CD40 and $\alpha 5\beta 1$ integrin on the fibroblasts. Flow cytometry analysis showed that CD40L expression was higher in the asthmatic T cells ($39.9 \pm 7.5\%$ of positive cells) than in T cells from the healthy controls ($15.6 \pm 1.8\%$ of positive cells) (Fig. 2A and B). We also evaluated the expression of CD40 and $\alpha 5\beta 1$ on bronchial fibroblasts from both the asthmatic and the healthy subjects. CD40 was weakly expressed by both the asthmatic and the healthy fibroblasts (data not shown), whereas the $\alpha 5\beta 1$ integrin was constitutively expressed on both the HF and the AF ($96.9 \pm 1.5\%$ vs $96.4 \pm 2.2\%$ of positive cells; $p = 0.6$) (Fig. 2C and D). There was no significant difference in mean fluorescence between the asthmatics and the healthy controls.

3.3. Effect of CD40L neutralization on IL-6 production by bronchial fibroblasts

T cells were pre-incubated with an anti-CD40L antibody prior to the co-culture with fibroblasts. CD40L neutralization reduced IL-6 expression to baseline levels in asthmatic fibroblasts co-cultured with T cells (4.8 ± 0.8 to 1.5 ± 0.6 -fold increase; $p = 0.001$), while this antibody did not affect T cell-induced fibroblast IL-6 expression

in healthy fibroblasts (4.1 ± 1.1 to 4.0 ± 1.6 -fold increase; $p = 0.96$) (Fig. 3).

3.4. CD40L ligation to $\alpha 5\beta 1$ receptors

To delineate the type of receptor involved in CD40L-induced IL-6 production by the fibroblasts, we performed a T cell/fibroblast

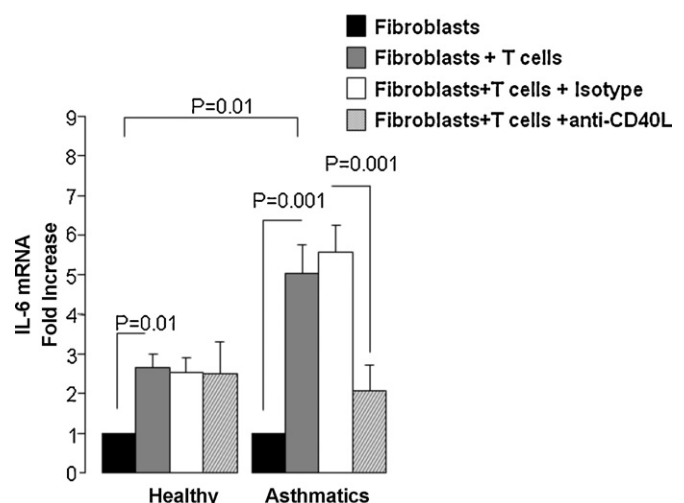


Fig. 3. Role of CD40L in the induction of IL-6 expression. Quantitative PCR analysis of IL-6 mRNA of fibroblasts co-cultured with T cells in the presence of anti-CD40L. Results are presented as the fold increase over fibroblast IL-6 mRNA expression in the absence of T cells. Data are presented as means $\pm$ SEM of six experiments.

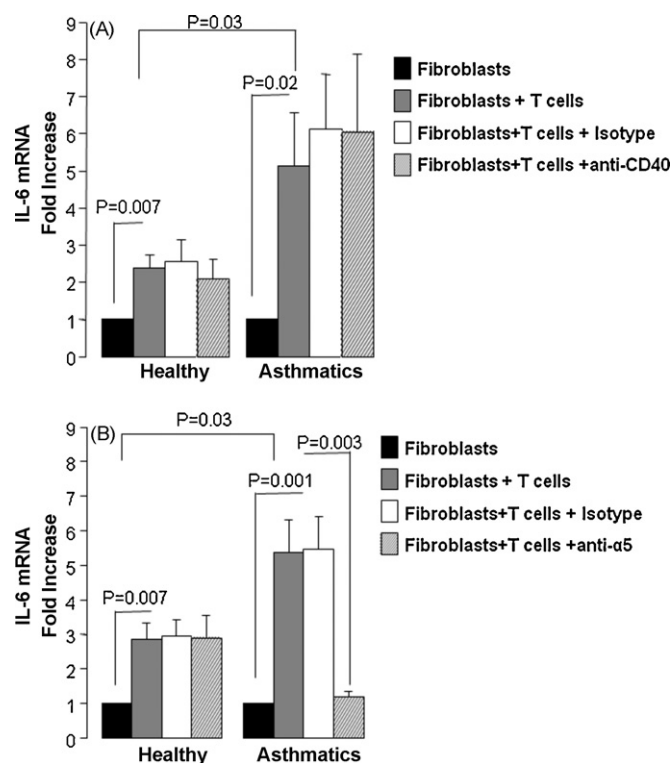


Fig. 4. Role of CD40 and $\alpha 5$ integrin on IL-6 expression. Quantitative PCR analysis of IL-6 mRNA of fibroblasts co-cultured with T cells in the presence of anti-CD40 mAb (A) and anti- $\alpha 5$ (B). Results are presented as the fold increase over fibroblast IL-6 mRNA expression in the absence of T cells. Data are presented as mean $\pm$ SEM of six experiments.

co-culture in the presence of anti-CD40 or anti- $\alpha 5$ antibodies. Anti-CD40 antibody did not inhibit IL-6 up-regulation in the healthy fibroblasts (2.7 ± 0.4 to 2.1 ± 0.5 -fold increase; $p = 0.9$) nor in the asthmatic fibroblasts (5.1 ± 1.4 to 6.0 ± 2.1 -fold increase; $p = 0.7$) co-cultured with T cells (Fig. 4A). However, anti- $\alpha 5$ antibody significantly inhibited T cell-induced IL-6 up-regulation in the asthmatic fibroblasts (5.4 ± 0.9 to 1.2 ± 0.2 -fold increase; $p = 0.003$) but not in the healthy fibroblasts (2.8 ± 0.5 to 2.9 ± 0.6 -fold increase; $p = 0.6$) (Fig. 4B).

3.5. Stimulation of fibroblasts with rsCD40L

To confirm the effect of CD40L on the fibroblasts, we stimulated the fibroblasts both from the asthmatics and the healthy subjects with trimeric recombinant soluble CD40L (rsCD40L). We found that rsCD40L stimulated IL-6 gene expression in the asthmatic but not in the healthy fibroblasts (7.5 ± 1.1 -fold increase; $p = 0.004$ vs 1.7 ± 0.6 -fold increase; $p = 0.47$; Fig. 5A). This stimulation was prevented by the pre-incubation of the fibroblasts with anti- $\alpha 5$ antibody (7.5 ± 1.1 -fold increase to 0.85 ± 0.6 -fold increase; Fig. 5A).

3.6. Binding assay

The binding assay of rsCD40L showed that rsCD40L only bound to asthmatic but not healthy fibroblasts. This binding was also blocked by the pre-incubation of the fibroblasts with an anti- $\alpha 5$ antibody (Fig. 5B).

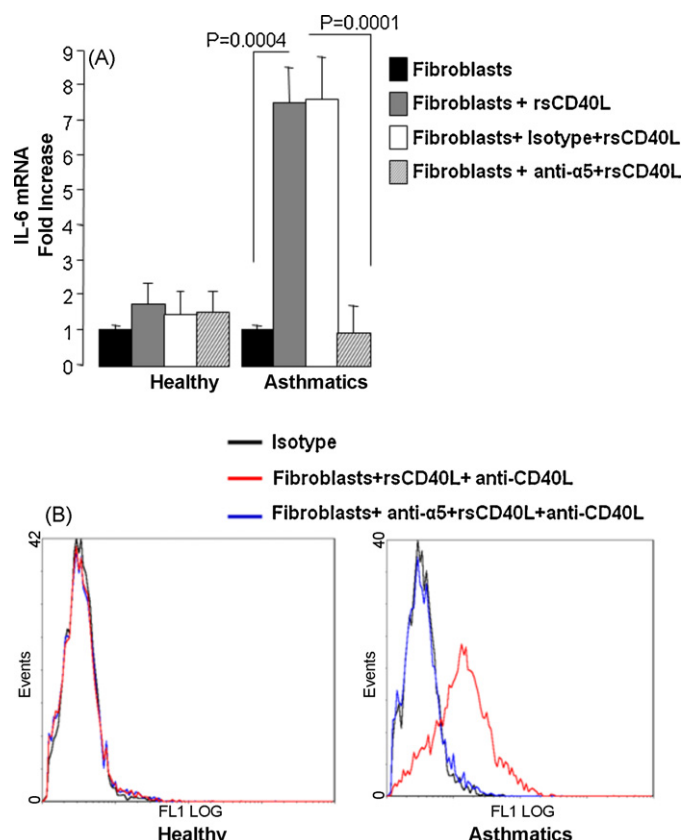


Fig. 5. rsCD40L binding assay. (A) Bronchial fibroblasts were treated or not with anti- $\alpha 5$ antibody and then stimulated with rsCD40L. (B) Flow cytometry histogram of rsCD40L binding test on fibroblasts with or without pre-treatment with anti- $\alpha 5$ antibody. Data for the mRNA results are presented as the fold increase over fibroblast IL-6 mRNA expression in the absence of T cells. Data are presented as mean $\pm$ SEM of six experiments.

3.7. Adhesion assay

The implication of $\alpha 5 \beta 1$ in IL-6 production by the asthmatic fibroblasts and not the healthy fibroblasts may be due to its different activated state in the different fibroblasts. To check this possibility, an adhesion test was performed. We found that the adherence to fibronectin, the ligand for $\alpha 5 \beta 1$, was more than two times higher for the asthmatic fibroblasts than for the healthy fibroblasts after 1 h (1.9 ± 0.4 O.D. vs 0.95 ± 0.2 O.D. at 580 nm; $p = 0.01$) of adhesion on a fibronectin layer (Fig. 6A). Anti- $\alpha 5$ antibody significantly reduced the adhesion for both the healthy (0.95 ± 0.2 O.D. vs 0.2 ± 0.1 O.D. at 580 nm; $p = 0.006$) and the asthmatic fibroblasts (1.9 ± 0.4 O.D. vs 0.6 ± 0.1 O.D. at 580 nm; $p = 0.007$) (Fig. 6A). Culture of fibroblasts on a fibronectin layer significantly up-regulated IL-6 gene expression in asthmatic fibroblasts only compared to culture on plastic (Fig. 6B).

4. Discussion

In this study, we show that interaction between T cells and bronchial fibroblasts; structural cells that play an important role in airway remodelling in asthma; leads to an increased production of IL-6 and that this interplay involved CD40L interaction with the integrin $\alpha 5 \beta 1$, rather than the classical CD40.

Inflammation in the airways is orchestrated by complex cellular and molecular interactions, which involve resident cells of the bronchial mucosa, as well as infiltrating inflammatory cells. Our findings showed that resident cells such as fibroblasts, by the release of soluble signals and/or direct interaction with other

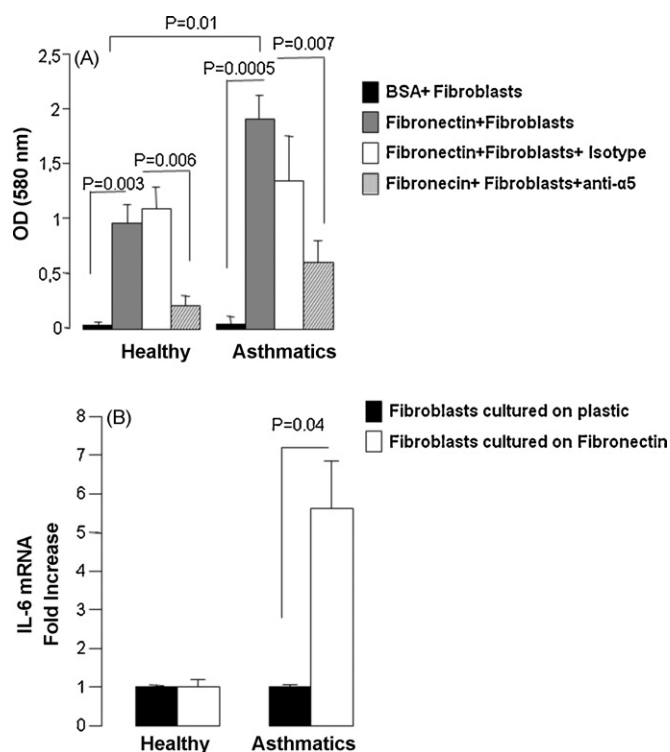


Fig. 6. Fibroblasts adhesion assay. (A) Healthy and asthmatic fibroblasts were left to attach in wells coated with either BSA or fibronectin with or without anti- $\alpha 5$ antibody for 1 h and cell adhesion was determined as described under Section 2. Results are presented as the optical density measured at 580 nm. (B) IL-6 mRNA expression by the bronchial fibroblasts following adhesion on fibronectin and plastic, respectively. Results are representative of six experiments and are presented as mean $\pm$ SEM.

cells, may serve as potential regulators of the local inflammatory response. Indeed, it has been reported that normal human lung fibroblasts co-cultured with LPS-activated monocytes modulate IL-10 and IL-12 production (Vancheri et al., 2001). We previously showed that fibroblasts derived from asthmatics have the capacity to increase IL-4 production by mast cells (Plante et al., 2006). Thus, the crosstalk between fibroblasts and inflammatory cells may play an important role in the pathogenesis of inflammation and airway remodelling.

We showed that the contact between T cells and bronchial fibroblasts led to a significant increase in IL-6. We have used IL-6 induction as readout for T cell–fibroblast interaction as IL-6 plays an important role in asthma tissue remodelling. Multiple lines of evidence indicate that IL-6 is a good marker of fibrosis. IL-6 expression and secretion has been shown to be correlated with fibrosis in tissues such as the liver, skin and lungs (Kayano and Okita, 2000; Sato et al., 2001; Smith et al., 1998). Furthermore, IL-6 knockout mice showed a significant reduction of fibrosis (Natsume et al., 1999). These previous studies support the important role of IL-6 in fibrogenesis. In addition, IL-6 is a key factor in shifting the Th1/Th2 balance towards Th2 direction and for inducing T cell activation and survival (Doganci et al., 2005; Chung and Barnes, 1999; Diehl and Rincon, 2002).

Based on the above experimental evidence, we set out to identify the nature of molecular interaction involved in IL-6 up-regulation following direct contact between T cells and fibroblasts. We tested different molecules such as ICAM-1, ICOSL and OX40L to find that blocking LFA-1/ICAM-1 interaction partially reduced (approximately 40% reduction) IL-6 production in asthmatic cells which only suggests that other molecules are involved (data not shown). We then looked at the expression of B7-1 and B7-2, ICOSL and

OX40L by bronchial fibroblasts and found that these cells expressed none of these molecules which exclude their involvement in the observed IL-6 increase (data not shown). Cell–cell crosstalk involved several molecules including interaction of CD40L with its receptors. In asthma, the CD40L/receptor dyad is thought to play a role in disease development as assessed by the inhibition of both pulmonary inflammation and fibrosis in the CD40L-deficient mouse model (Lei et al., 1998; Kowal et al., 2006). We then performed T cell/fibroblast co-cultures in the presence of a blocking anti-CD40L antibody. We found that IL-6 production was completely abolished in asthmatic fibroblasts. This suggests that this IL-6 up-regulation in asthmatic fibroblasts is mediated through CD40L ligation with its receptors. T cells obtained from asthmatics expressed CD40L suggesting that these cells were activated. A subsequent co-culture in the presence of an anti-CD40 showed no effect on IL-6 up-regulation on both the healthy and the asthmatic fibroblasts. In fact, these cells displayed very low CD40 expression, which suggests that CD40L may interact more with other receptors. Recent data showed that CD40L can interact with $\alpha 5 \beta 1$ (VLA-5) (Leveille et al., 2007). As reported above, the bronchial fibroblasts expressed this integrin. We thus performed a T cell/fibroblast co-culture in the presence of a neutralizing anti- $\alpha 5$ antibody which showed a complete inhibition of T cell-induced IL-6 up-regulation solely in the asthmatic fibroblasts. Therefore, the existence of a significantly increased CD40L expression in asthmatic T cells compared with healthy T cells, as shown here, may enhance the possibility of binding with the constitutively expressed $\alpha 5 \beta 1$ on the fibroblasts, thus explaining both anti-CD40L and anti- $\alpha 5$ blocking effects on IL-6 up-regulation in the asthmatic and not in the healthy ones. Furthermore, these results may also be explained by the existence of the integrin $\alpha 5 \beta 1$ in different states of activation in asthmatic as opposed to healthy subjects. Integrin-mediated adhesion is a function of cell-surface integrin density, activation state, and ligand density. Thus, the cell adhesion assay reveals that asthmatic fibroblast adherence to fibronectin is increased compared to that of healthy fibroblasts. The increased adhesion to fibronectin observed in the asthmatic fibroblasts suggests that $\alpha 5 \beta 1$ integrin may exist in different activation states, with a high affinity state in asthmatic patients and a low affinity state in healthy patients. To support this hypothesis, several studies showed that in resting cells, integrins exist in a low affinity conformation, and in order to induce adhesion, a conformational change to high affinity state is required (van der and Sonnenberg, 2001). Several cytokines and chemokines that have been shown to be increased in the asthmatic bronchial tissues, such as IL-8 and MCP-1 (Kowal et al., 2006; Ashida et al., 2001), may promote $\alpha 5 \beta 1$ to a higher activation state by intracellular signalling, referred to as “inside out signalling” (Lloyd et al., 1996; Ashida et al., 2001; Harburger and Calderwood, 2009). This higher activation state then allows an increased fibroblast adhesion to fibronectin as well as an increased CD40L ligation from T cells, as this last result in a stimulation of IL-6. Therefore, the exclusivity of the CD40L/ $\alpha 5 \beta 1$ role to asthmatic fibroblasts could be attributed to the fact that inflammatory events observed in asthma may enhance CD40L/ $\alpha 5 \beta 1$ interaction by mechanisms that respectively affect the activation state of the $\alpha 5 \beta 1$ integrin and the up-regulation of the CD40L expression in T cells.

In conclusion, T cell/fibroblasts interaction that may have an impact on airway remodelling is triggered via the CD40L/ $\alpha 5 \beta 1$ interaction with fibroblasts. This study supports the concept that resident cells such as fibroblasts act as key cell mediators of airway inflammation and remodelling, as they produce various cytokines involved in leukocyte recruitment, and the airway fibrotic process.

Declaration of all sources funding: Lionel Loubaki received a grant from the training program in respiratory health of the Fonds de la Recherche en Santé du Québec and the Canadian Institutes of Health Research and has no relationships to declare. Abdel-

habib Semlali has no relationships to declare. Eric Jacques has no relationships to declare. Sophie Plante has no relationships to declare. Walid Mourad received a grant from the Canadian Institutes of Health Research. Jamila Chakir received a grant from the Fonds J. Alphonse l'Espérance and the Canadian Institutes of Health Research.

Acknowledgments

We thank Drs. Julie Milot, Michel Laviolette of the Institut Universitaire de Cardiologie et de Pneumologie de Québec for the bronchoscopy procedures, Françoise Deschênes and Joanne Milot for blood collection, Haydar Alturaihi for technical advice and Serge Simard for statistical analysis. We thank all volunteers for their participation in this study and the Banque de Tissus du Réseau en Santé Respiratoire du FRSQ, Institut Universitaire de Cardiologie et de Pneumologie de Québec.

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