

Mast cell adhesion to bronchial smooth muscle in asthma specifically depends on CD51 and CD44 variant 6

P.-O. Girodet^{1,2,3}, A. Ozier^{1,2,4}, T. Trian^{1,2}, H. Begueret^{1,2,4}, O. Ousova^{1,2}, J.-M. Vernejoux⁴, P. Chanez⁵, R. Marthan^{1,2,4}, P. Berger^{1,2,4} & J. M. Tunon de Lara^{1,2,4}

¹Université de Bordeaux, Bordeaux, France; ²INSERM U885, Bordeaux, France; ³CHU de Bordeaux-INSERM, Centre d'Investigation Clinique, Bordeaux, France; ⁴CHU de Bordeaux, Bordeaux, France; ⁵Université de la Méditerranée AP-HM, Marseille, France

To cite this article: Girodet P-O, Ozier A, Trian T, Begueret H, Ousova O, Vernejoux J-M, Chanez P, Marthan R, Berger P, Tunon de Lara JM. Mast cell adhesion to bronchial smooth muscle in asthma specifically depends on CD51 and CD44 variant 6. *Allergy* 2010; DOI:10.1111/j.1398-9995.2009.02308.x.

Keywords

adhesion molecules; asthma; inflammation; mast cells; smooth muscle.

Correspondence

Pierre-Olivier Girodet, MD, PhD, INSERM U885, Université de Bordeaux, CHU de Bordeaux-INSERM, Centre d'Investigation Clinique, 146 rue Léo Saignat, F-33076 Bordeaux cedex, France.
Tel.: + 33 (0) 5 57 57 15 60
Fax: + 33 (0) 5 57 57 46 71
E-mail: pierre-olivier.girodet@pharmaco.u-bordeaux2.fr

Accepted for publication 23 November 2009

DOI:10.1111/j.1398-9995.2009.02308.x

Edited by: Marc Humbert

Abstract

Background: Mast cells infiltrate the bronchial smooth muscle (BSM) in asthmatic patients, but the mechanism of mast cell adhesion is still unknown. The adhesion molecules CD44 (i.e. hyaluronate receptor) and CD51 (i.e. vitronectin receptor) are widely expressed and bind to many extracellular matrix (ECM) proteins. The aims of the study are (i) to identify the role of ECM in mast cell adhesion to BSM and (ii) to examine the role of CD51 and CD44 in this adhesion.

Methods: Human lung mast cells, human mast cell line (HMC-1), and BSM cells from control donors or asthmatic patients were cultured in the presence/absence of various cytokines. Mast cell–BSM interaction was assessed using ³H-thymidine-pulsed mast cells, confocal immunofluorescence, or electron microscopy. Adhesion molecules expression and collagen production on both cell types were evaluated by quantitative RT-PCR, western blot, and flow cytometry.

Results: Mast cell adhesion to BSM cells mostly involved type I collagen of the ECM. Such an adhesion was increased in normal BSM cells under inflammatory condition, whereas it was maximal in asthmatic BSM cells. Blockade of either CD51 or CD44 significantly decreased mast cell adhesion to BSM. At the molecular level, protein and the transcriptional expression of type I collagen, CD51 or CD44 remained unchanged in asthmatic BSM cells or in mast cells/BSM cells under inflammatory conditions, whereas that of CD44 variant isoform 6 (v6) was increased.

Conclusions: Mast cell–BSM cell adhesion involved collagen, CD44, and CD51, particularly under inflammatory conditions. CD44v6 expression is increased in asthmatic BSM cells.

Asthma is a very frequent disease characterized by bronchial hyperresponsiveness, chronic bronchial inflammation, and remodeling (1). Mast cells have been shown to specifically infiltrate the asthmatic bronchial smooth muscle (BSM) (2–8). Such an infiltration involves the direct chemotactic activity of BSM cells for mast cells through the production

of transforming growth factor-beta 1 (TGF-β1), stem cell factor (SCF) (4), CXCL10 (5), and CX₃CL1 (6). Collectively, these findings suggest the presence of an auto-activation loop involving mast cell and BSM cell in asthma (4). The adhesion between both cell types may contribute to long-term effects leading to hyperresponsiveness and airway remodeling but its mechanism(s) remain poorly understood.

A direct cell–cell contact between mast cell and BSM cell has been initially suggested as an enhancing factor of mast cell degranulation, although evidence of a real adhesion between these cell types was not provided (9). Mast cells can really adhere to BSM cells, as demonstrated later by Yang et al. (10), implicating tumor suppressor in lung cancer-1 (TSLC-1) but for less than 30% of the adhesion (10).

Abbreviations

BSA, bovine serum albumine; BSM, bronchial smooth muscle; CD44v, CD44 variants isoform; ECM, extracellular matrix; FN, fibronectin; HLMC, human lung mast cell; PMA, phorbol 12-myristate 13-acetate; RANTES, regulated upon activation normal T cell expressed and secreted; SCF, stem cell factor; TGF-β1, transforming growth factor-beta 1.

Alternative mechanisms may thus be implicated in mast cell adhesion of the BSM. Such an adhesion can promote the survival and the proliferation of mast cells (11), leading to a prolonged BSM activation by mast cell mediators as found in asthmatic patients.

In this respect, we focused our attention on cell interaction with the extracellular matrix (ECM) through the CD51/CD61 or CD44 molecules for the following reasons. The heterodimer CD51/CD61 or $\alpha V/\beta 3$ also called vitronectin receptor belongs to the integrin family and has been associated with asthma phenotypes (12). CD51 is expressed in inflammatory cells including human mast cells (13, 14). In association with CD61, CD51 binds many ECM proteins such as vitronectin, fibronectin (FN), or collagen (15). The adhesion molecule CD44 has also been associated with asthma (16). Moreover, in a murine model of asthma, Katoh et al. have inhibited both bronchial hyperresponsiveness and lung inflammation using a blocking anti-CD44 Ab (17, 18). Finally, CD44 is a class I transmembrane glycoprotein originally described in leukocytes and expressed in most cell types including BSM cells and mast cells (19, 20). For instance, adhesion of T lymphocytes to the BSM implicates CD44 (21). Various ECM compounds including hyaluronic acid, collagen, laminin, and FN are ligands for CD44 (22). However, several CD44 variant isoforms (CD44v) are produced upon alternative splicing (23). Previous studies have shown that different variants are implicated in inflammatory diseases (v6) or allergic hypersensitivity (v7) (24), but their role in asthma remains to be elucidated, particularly in mast cell–BSM cell interaction.

The aims of the present study were thus (i) to identify the role of ECM in mast cell adhesion to BSM cell and (ii) to examine the role of CD51, CD44 and particularly that of CD44 variant isoforms in this adhesion.

Methods

A complete description of all Methods information is available in the Online Repository.

Study population and human tissue collection

A total of 11 patients with severe persistent asthma and 35 nonasthmatic controls were prospectively recruited from the 'Centre Hospitalier Universitaire de Bordeaux' according to the Global Initiative for Asthma guidelines (25) (Table 1). All subjects gave their written informed consent to participate in the study after the nature of the procedure had been fully explained. This study followed recommendations outlined in the Helsinki Declaration and received approval from the local ethics committee. Bronchial specimens from all subjects were obtained by either fiberoptic bronchoscopy or lobectomy, as previously described (4, 6, 26).

Cell culture and challenge

BSM cells were dissected as described previously (27). Human lung mast cells (HLMC) were dispersed from macro-

Table 1 Clinical and functional characteristics of subjects

Characteristics	Patients with asthma	Controls
Number of patients	11	35
Age (yr)	52.6 $\pm$ 4.3	63.4 $\pm$ 1.8
BMI (kg/m ²)	28.9 $\pm$ 1.1	24.3 $\pm$ 0.8
Smoking history		
Pack years	0 $\pm$ 0	40.5 $\pm$ 6.0
Current (number of patients)	0	14
Former (number of patients)	0	21
Years since quitting	–	13.1 $\pm$ 6.0
Treatments		
LABA (number of patients)	7	3
ICS (number of patients)	11	3
OCS (number of patients)	5	0
FEV ₁		
Liters	1.7 $\pm$ 0.4	2.5 $\pm$ 0.1
Percentage of predicted value	66.1 $\pm$ 10.8	82.5 $\pm$ 3.7
FEV ₁ : FVC ratio (percentage of FVC)	69.9 $\pm$ 6.6	69.0 $\pm$ 2.6
FEF 25-75		
Liters per second	1.5 $\pm$ 0.4	2.1 $\pm$ 0.3
Percentage of predicted value	44.1 $\pm$ 12.2	62.2 $\pm$ 7.9

Data are the mean $\pm$ the SEM. BMI, body mass index; LABA, long-acting β -2 agonist; ICS, inhaled corticosteroid; OCS, oral corticosteroid; FEV₁, forced expiratory volume in one-second; FVC, forced vital capacity; FEF 25-75, forced expiratory flow between 25 and 75% of FVC.

scopically normal lung and then purified using anti-CD117-coated immunomagnetic beads (Dynal collection kit; Dynal, Oslo, Norway) as previously described (28, 29). BSM cells or the human mast cell line HMC-1 (30) was cultured in culture medium DMEM (Invitrogen, Cergy Pontoise, France) containing 10% fetal calf serum. Isolated HLMC were cultured in the same medium containing 100 ng/ml SCF, 50 ng/ml IL-6, and 10 ng/ml IL-10 (all from R&D Systems, Abingdon Oxon, UK) as described previously (28).

Cell purity was assessed by both immunocytochemistry and flow cytometry (Beckton Dickinson, Pont de Claix, France). Adhesion molecules expression on mast cells or BSM cells was also assessed by flow cytometry. Briefly, cells were fixed with 4% paraformaldehyde (Thermo Fisher Scientific, Illkirch, France) and incubated for 30 min on ice with mouse Abs against human CD51 (Beckman Coulter, Roissy CDG, France), panCD44 (Dako, Trappes, France) or CD44v4, v6 or v9 (20) or isotype control Abs. Cells were analyzed for their fluorescence intensity using FACS Calibur (Beckton Dickinson). Results are expressed as mean fluorescence intensity (MFI) or percentage of positive cells.

BSM cells were stimulated with optimal concentrations of IL-1 β , TNF- α , IL-6 (all from R&D Systems), or SLIGKV-NH₂ (Sigma-Aldrich, Saint Quentin-Fallavier, France), the agonist peptide of protease activated receptor 2 (PAR-2), for 72 h. Mast cells were challenged with optimal concentrations of TGF- β 1 (transforming growth factor-beta 1), SCF (stem cell factor), RANTES (regulated upon activation normal

T cell expressed and secreted), fractalkine (all from R&D Systems), PMA (phorbol 12-myristate 13-acetate) or calcium ionophore A23187 (both from Sigma-Aldrich) for 1 h at 37°C.

Mast cell-BSM cell adhesion

HMC-1 or HLMC adhesion to BSM cells was initially assessed using radiolabeled mast cells as adapted from Lazaar et al. (21) using pulsed mast cells with 10 $\mu\text{Ci/ml}$ [^3H]-thymidine (Amersham Biosciences, Orsay, France).

In another set of experiments, mast cells were allowed to adhere to a monolayer of BSM cells for 1 h at 37°C in 8-well chamber slides or cell culture inserts (VWR international, Strasbourg, France). Mast cells and BSM cells were stained respectively with Texas Red[®]-conjugated anti-tryptase (Dako) and FITC-conjugated anti- α -smooth muscle actin Abs (Molecular Probes, Eugene, OR, USA). Confocal images were obtained using Fluoview laser scanning microscope (Nikon, Paris, France) and reconstituted in 3D images using IMARIS Software (Bitplane, Zürich, Switzerland) as described previously (6). Electronic images from inserts were obtained using Philips 301 electron microscope (Philips Electron Optics, Eindhoven, The Netherlands) (7).

RNA extraction, reverse transcription, and real-time quantitative PCR

Total RNA was extracted from BSM cells and mast cells as described previously (31). After reverse transcription, real-time quantitative PCR was performed on a Rotor-Gene[™] 2000 (Corbett Research, Mortlake, Sydney, Australia), using appropriate primers (Sigma-Aldrich) designed to target CD51, CD44 or collagen type I $\alpha 1$. Results were normalized by the geometric mean of three internal housekeeping genes (32).

Immunoblotting

Western blot was performed on cell protein extracts (26) using primary antibody directed against collagen type I α monoclonal Ab (Santa Cruz, Heidelberg, Germany). Blot images were acquired using BIOCAPTMW software (Thermo Fisher Scientific). Band densities were quantified using IMAGEJ software and normalized to that of β -actin.

Statistical analysis

Values are presented as the mean $\pm$ the SEM. Statistical analysis was performed using NCSS 2001 software. Statistical significance was analyzed by ANOVA, Kruskal-Wallis and paired Student's *t*-test. *P*-value <0.05 was taken as the threshold for statistical significance.

Results

Human mast cells adhere to human BSM

We have observed that mast cells adhered to a monolayer of BSM cells (Fig. 1A). This adhesion was significantly higher

Mast cell adhesion to bronchial smooth muscle

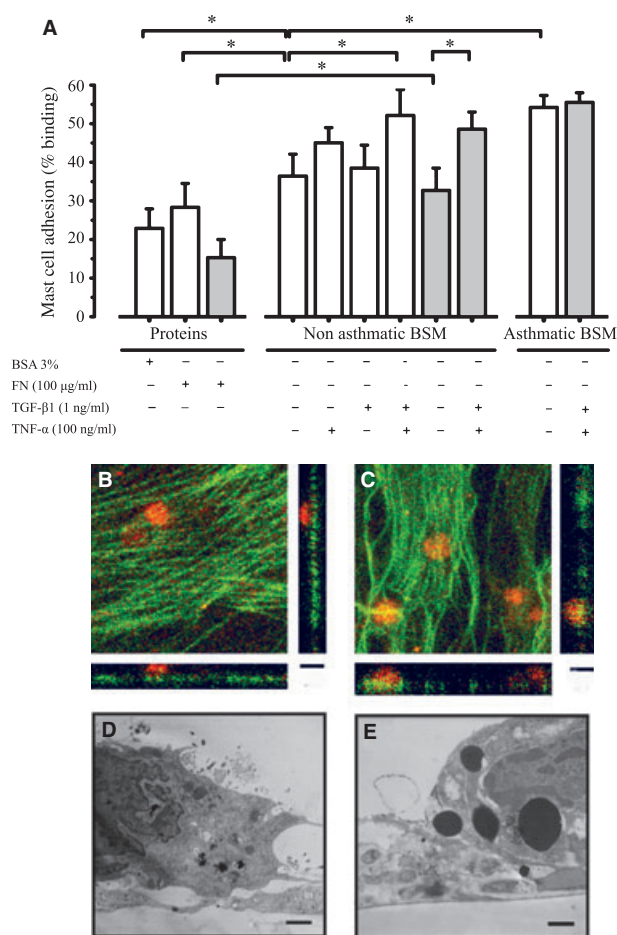


Figure 1 Mast cell-bronchial smooth muscle (BSM) cell adhesion. (A) [^3H]-mast cells adhere to proteins, nonasthmatic or asthmatic BSM cells. Adhesion was performed using HMC-1 (white bars; $n = 10$) or human lung mast cell (grey bars; $n = 6$). Mast cells were not stimulated (-) or stimulated (+) with 1 ng/ml transforming growth factor-beta 1 (TGF- $\beta 1$) for 1 h; BSM cell were not stimulated (-) or stimulated (+) with 100 ng/ml TNF- α for 72 h. Data are means $\pm$ SEM. *, $P < 0.05$. (B and C) Representative confocal images presented as three axis slices demonstrating tryptase-positive mast cells (red) adhesion to α -smooth muscle actin-positive BSM cells (green). Bars represent 20 μm . (D and E) Representative electronic microscopic images of HMC-1 adhesion to nonasthmatic BSM cells. Bars represent 1 μm . Both mast cells and BSM cells were not stimulated (B and D) or stimulated with 1 ng/ml TGF- $\beta 1$ for 1 h and 100 ng/ml TNF- α for 72 h, respectively (C and E).

than that of mast cells to either bovine serum albumine (BSA) or FN. Among a variety of cytokines and chemokines that can stimulate these two cell types, the concomitant stimulation of mast cells by TGF- $\beta 1$ and BSM cells by TNF- α was the main factor able to significantly increase this adhesion (Table 2). Analysis of time course and concentration effect indicated that adhesion was maximal following stimulation of BSM cells with 100 ng/ml TNF- α for 72 h and

HMC-1 cells with 1 ng/ml TGF- β 1 for 1 h. Similar results were obtained using either HLHC or HMC-1 for the adhesion on BSM cells (Fig. 1A). In addition, in the absence of any stimulation, mast cell adhesion was significantly increased using BSM cells from asthmatic patients when compared to that using nonasthmatic BSM cells (Fig. 1A).

Confocal microscopy and double staining immunofluorescence confirmed that mast cells adhered to BSM cells (Fig. 1B) and that, upon stimulation, the number of adherent mast cells increased (Fig. 1C). Using electron microscopy performed under identical experimental conditions, a close interaction between mast cells and BSM cells was also visualized (Fig. 1D, E). However, we were unable to observe either direct cell-cell contact or tight junctions, whatever the stimulation state.

Human mast cells adhere to BSM through CD51 and CD44

To investigate the mechanism of mast cell adhesion to BSM, we first evaluated the effect of blocking Abs against intercellular adhesion molecules. Blocking ICAM-1 (CD54), VCAM-1 (CD106), CD40L (CD154), c-kit (CD117), or fractalkine (CX3CL1) had no effect on spontaneous or stimulated mast cell adhesion to BSM cell (Table S1 in the Online Repository).

We, then, investigated the role of mast cell and BSM cell receptors for ECM proteins. Blocking the mast cell α -v subunit of vitronectin receptor (CD51) slightly decreased mast cell adhesion to stimulated BSM cells, whereas blocking BSM CD51 had no effect (Fig. 2A). Similar results were obtained using either HMC-1-stimulated or HLHC-stimulated cells. However, in unstimulated cells, blocking CD51 was efficient only in HMC-1. Moreover, blocking BSM or mast cell transmembrane glycoprotein CD44 significantly decreased the adhesion of stimulated cells indicating that CD44 is implicated in both cell types (Fig. 2B). In addition, blocking CD44 simultaneously in both mast cells and BSM cells had a synergistic effect. Again, similar results were obtained with either HMC-1 or HLHC.

To investigate this further, we quantified CD51 and CD44 mRNA using real-time RT-PCR (Fig. 2C). Although CD51 mRNA levels remained unchanged in both cell types following the appropriate stimulation, we observed a transient elevation of CD44 mRNA level after 6 h in TNF- α -stimulated BSM cells. However, this transcriptional effect was not associated with an increase in CD44 protein expression assessed by flow cytometry (Fig. 2D). Indeed, CD44 was highly expressed at the surface of nonasthmatic BSM cells (MFI: 235.3 ± 58.8 ; percentage of positive cells: 97.8 ± 0.7 ; $n = 6$), asthmatic BSM cells (MFI: 242.2 ± 31.4 ; % of positive cells: 98.1 ± 0.9 ; $n = 6$), and mast cells (MFI: 60.2 ± 6.6 ; percentage of positive cells: 91.2 ± 5.0 ; $n = 6$) under baseline conditions.

Mast cell adhesion to BSM involves collagen type I α 1 but not hyaluronic acid

As blocking CD44 specifically inhibited mast cell adhesion enhanced by TGF- β 1 and TNF- α , we analyzed the role of CD44 ligands in mast cell adhesion to BSM. A pretreatment of BSM with hyaluronidase was used to evaluate the role of hyaluronic acid, the main ligand of CD44. Surprisingly, mast cell adhesion was not affected by such a pretreatment (data not shown). By contrast, previous incubation of BSM cells monolayer with type I collagenase abolished mast cell adhesion, whereas the same treatment had no effect on mast cell adhesion to FN (Fig. 3A).

It is noteworthy that such inhibitory effect was observed in both stimulated and nonstimulated cells. As adhesion was upregulated by TGF- β 1 and TNF- α , we investigated whether these cytokines increase the production of collagen type I. TGF- β 1 and TNF- α did not stimulate collagen synthesis by mast cells (data not shown) and BSM cells, respectively, at both transcriptional (Fig. 3B) and protein levels (Fig. 3C). Comparison between mRNA and protein collagen type I production by BSM cells obtained from control donors ($n = 5$) and asthmatic patients ($n = 3$) revealed no significant difference (Fig. 3D, E).

Table 2 Modulation of human mast cell adhesion to BSM cell

Mast cell challenge (1 h)	BSM cell challenge (72 h)				
	Nonstimulated	TNF- α (100 ng/ml)	IL-1 β (100 ng/ml)	IL-6 (100 ng/ml)	SLIGKV (10^{-4} M)
Nonstimulated	34.7 ± 2.7	39.1 ± 6.8	40.5 ± 10.9	42.6 ± 12.4	26.6 ± 6.3
TGF- β 1 (1 ng/ml)	36.8 ± 5.5	$49.1 \pm 4.5^*$	35.9 ± 6.0	34.6 ± 4.3	22.7 ± 5.1
SCF (30 ng/ml)	29.7 ± 9.0	37.5 ± 8.1	34.7 ± 11.6	32.4 ± 9.4	34.1 ± 12.4
RANTES (100 ng/ml)	30.1 ± 3.2	31.2 ± 4.7	27.4 ± 3.8	32.5 ± 6.0	34.1 ± 6.6
Fractalkine (50 ng/ml)	30.3 ± 5.4	34.1 ± 3.8	37.7 ± 3.2	31.1 ± 3.2	29.3 ± 5.1
A23187 (200 nM)	31.4 ± 8.3	36.4 ± 5.7	30.6 ± 8.0	32.4 ± 5.1	34.7 ± 2.9
PMA (40 nM)	17.3 ± 2.9	33.2 ± 8.8	21.0 ± 4.0	20.1 ± 6.7	22.1 ± 6.0

PMA, phorbol 12-myristate 13-acetate; RANTES, regulated upon activation normal T cell expressed and secreted; SCF, stem cell factor; TGF- β 1, transforming growth factor-beta 1.

Mast cells labelled with [^{3} H] thymidine (10 μ Ci/ml) adhered for 1 h to culture plates coated with growth-arrested bronchial smooth muscle (BSM) cells. Values are expressed relative to maximal adhesion. Data are means $\pm$ SEM of six independent experiments. * $P < 0.05$ vs non-stimulated conditions, paired Student's t -test.

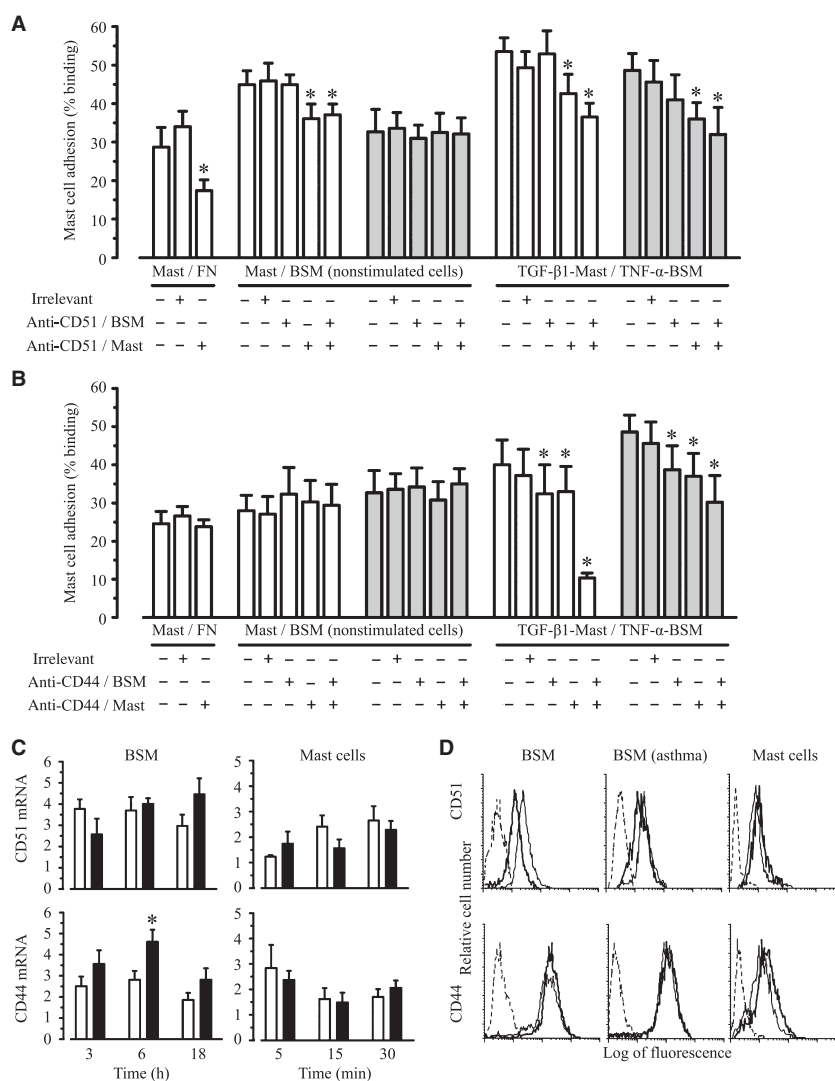


Figure 2 Roles of CD51 and CD44 in mast cell-bronchial smooth muscle (BSM) cell adhesion. (A and B) Effect of anti-CD51 (A) or anti-CD44 (B) blocking Ab on mast cell–BSM adhesion. [^{3} H]-mast cells adhere to fibronectin or nonasthmatic BSM cells. Adhesion was performed using HMC-1 (white bars; $n = 10$) or human lung mast cell (grey bars; $n = 6$). Mast cells were not stimulated or stimulated with 1 ng/ml transforming growth factor-beta 1 (TGF- β 1) for 1 h; BSM cells were not stimulated or stimulated with 100 ng/ml TNF- α for 72 h. Data are means $\pm$ SEM. *, $P < 0.05$ vs irrelevant Ab conditions.

Role of CD44 variant isoforms in mast cell–BSM cell adhesion

As, on the one hand, adhesion of mast cells to BSM cells was increased using asthmatic BSM cells compared to nonasthmatic cells or using stimulated cells compared to nonstimulated cells, and, on the other hand, neither collagen production nor CD44 expression was increased under these experimental conditions, we investigated the expression of several CD44 variant isoforms. Using flow cytometry, we

analyzed the expression of CD44v4, CD44v6, and CD44v9, which are all expressed in lung tissue (16). Stimulation of BSM cells by TNF- α increased the expression of CD44v6 (MFI: 28.1 ± 1.7 vs 12.3 ± 2.5 , $P = 0.02$; Fig. 4A), decreased that of CD44v4 (13.7 ± 2.6 vs 24.6 ± 1.9 , $P = 0.03$; Fig. 4B), and left unchanged that of CD44v9 (14.2 ± 1.5 vs 14.7 ± 2.3 , $P = 0.3$; Fig. 4C). By contrast, stimulation of asthmatic BSM cells by TNF- α did not alter the expression of these variant isoforms (Fig. 4D–F). However, CD44v6 was constitutively upregulated in asthmatic

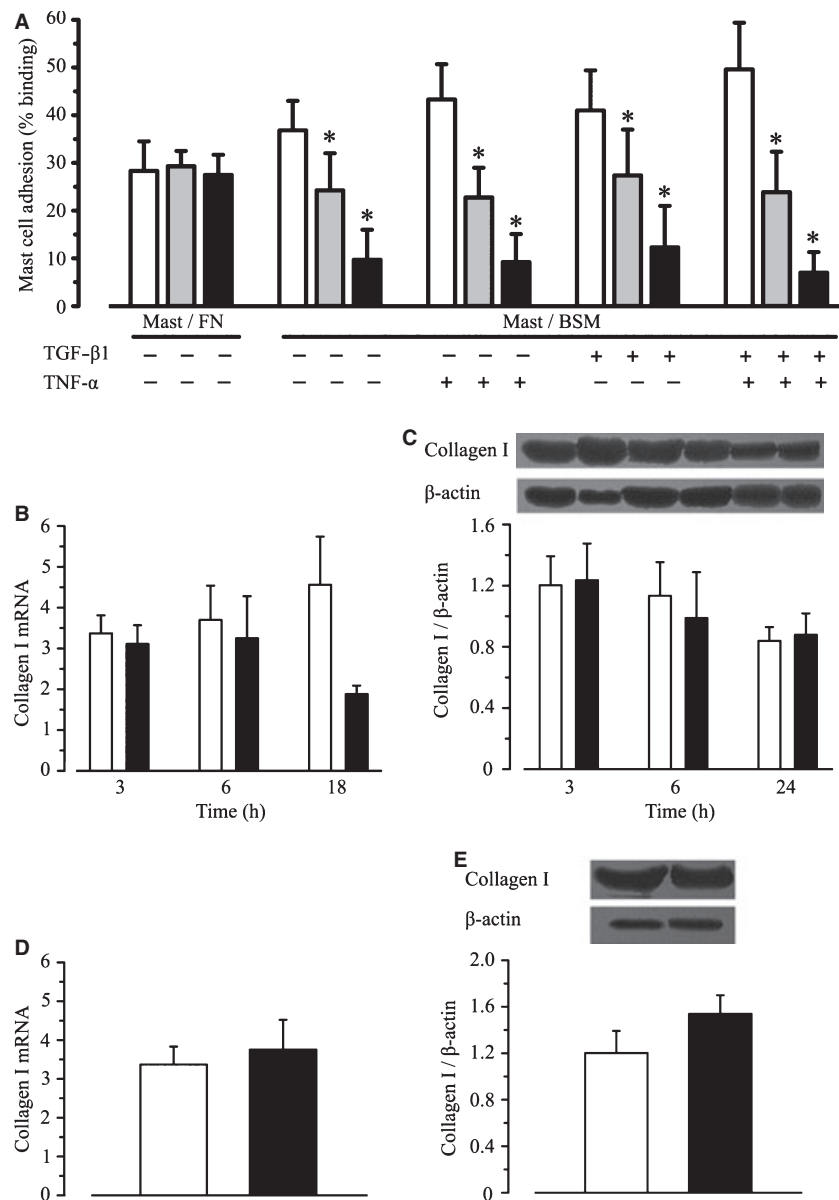


Figure 3 Collagen type I mediates mast cell-bronchial smooth muscle (BSM) cell adhesion. (A) [^{3} H]-HMC-1 cells adhere to fibronectin or nonasthmatic BSM cells. Mast cells were not stimulated (-) or stimulated (+) with 1 ng/ml transforming growth factor-beta 1 for 1 h; BSM cells were not stimulated (-) or stimulated (+) with 100 ng/ml TNF- α for 72 h. Adhesion was inhibited by 0.1 μ g/ml (grey bars) and 1 μ g/ml (black bars) collagenase

($n = 8$). Data are means $\pm$ SEM. *, $P < 0.05$ vs condition without collagenase. (B-E) Collagen type I $\alpha 1$ level on BSM cells was assessed by quantitative RT-PCR (B and D, $n = 5$) and Western blot (C and E, $n = 5$). (B and C) Cells were either nonstimulated (white bars) or stimulated with 100 ng/ml TNF- α for 72 h. (D and E) BSM cells from control donors (white bars) or asthmatic patients (black bars).

BSM cells (MFI: 30.0 ± 2.4 vs 12.3 ± 2.5 , $P = 0.02$; Fig. 4A, D). Stimulation of mast cells by TGF- $\beta 1$ also increased the expression of CD44v6 (MFI: 43.7 ± 4.1 vs 22.6 ± 3.2 , $P = 0.001$; Fig. 4G) but not that of isoforms v4 and v9 (12.4 ± 3.8 vs 14.2 ± 4.4 , $P = 0.3$ and 31.0 ± 2.9 vs 30.5 ± 4.1 , $P = 0.5$, respectively; Fig. 4H, I). Taken together, these results demonstrated that CD44v6 is upregulated in inflammatory experimental conditions in both mast

cells and BSM cells, particularly in that of asthmatics. Such findings suggest that CD44v6 may be an important factor for mast cell adhesion to asthmatic BSM.

Discussion

In the present study, we have demonstrated that mast cells adhere to the BSM mainly via type I collagen of the ECM.

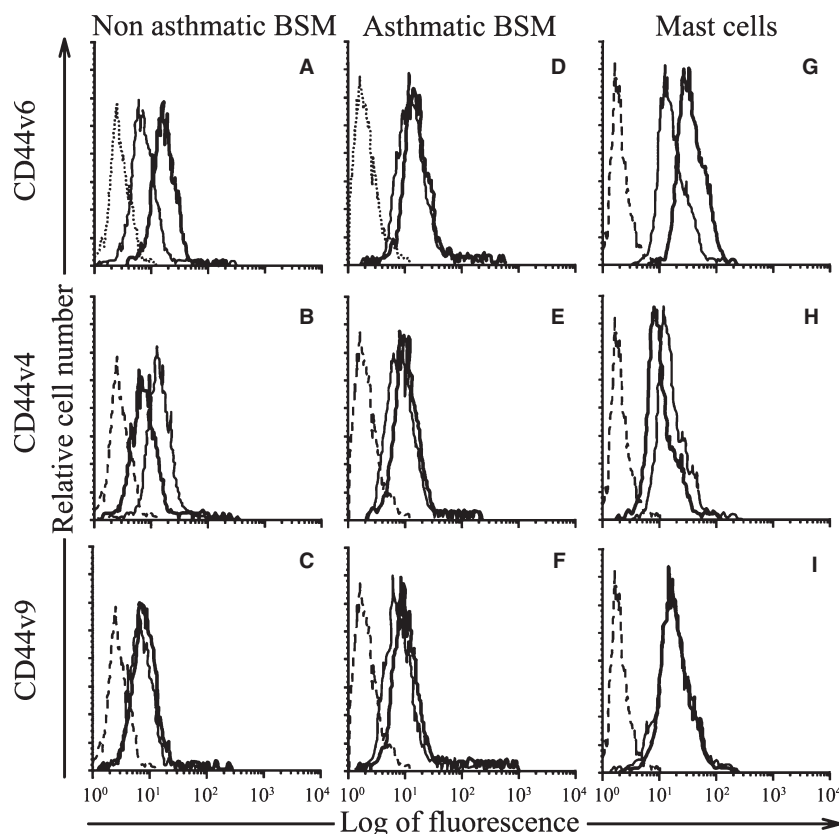


Figure 4 CD44v6 is specifically upregulated in bronchial smooth muscle (BSM) cells and mast cells. FACS representative histograms were obtained using nonasthmatic BSM cells (A, B, C) asthmatic BSM cells (D, E, F) or mast cells (G, H, I). CD44v6 (A, D, G), CD44v4 (B, E, H) or CD44v9 (C, F, I) expression was

assessed using irrelevant Ab (dotted lines) or specific Abs (full lines). Cells are either nonstimulated (fine lines) or stimulated (thick lines) with 1 ng/ml transforming growth factor-beta 1 for 1 h (mast cells) or 100 ng/ml TNF- α for 72 h (BSM cells).

This adhesion is reinforced under inflammatory conditions in normal BSM cells, whereas it is maximal in asthmatic BSM cells, and involves both CD51 and CD44. The increased adhesion of mast cell to asthmatic BSM cells was associated with an increased expression of CD44v6. To the best of our knowledge, this is the first description of the molecular mechanism implicated in such adhesion. This mechanism may account for the very specific mast cell infiltration in the smooth muscle layer of bronchi in asthma (2, 4).

Whereas it has been reported that mast cell adhere to BSM (9–11), the role of ECM has not been investigated so far. In the present study, we demonstrate, for the first time, that the main mechanism of mast cell adhesion to BSM involves ECM proteins which are present at the periphery of myocytes. This cell/matrix/cell interaction may generate a co-stimulatory signal for mast cell activation (33) that, in turn, leads to inflammatory mediators release playing a role in smooth muscle contraction and proliferation (34, 35). Such an interaction may implicate different components of the ECM, although type I collagen appears to be the main ECM protein involved. Indeed, adhesion of

mast cells to BSM was decreased by type I collagenase to a low level compared to that of spontaneous nonspecific adhesion to BSA.

In addition, we were able to increase the mast cell adhesion to BSM under inflammatory experimental conditions mimicking asthmatic bronchial inflammation. For instance, TNF- α also increased BSM-induced mast cell chemotaxis (4). Interestingly, mast cell adhesion was also increased using asthmatic BSM cells. Indeed, asthmatic BSM cells retain several pathophysiological characteristics *in vitro*, such as increased proliferation (26, 36, 37) or increased mast cell chemotaxis (5).

We paid special attention to CD51 and CD44, because both receptors recognize type I collagen (38, 39). Blocking either CD51 or CD44 significantly decreased mast cell adhesion to BSM, thus indicating that both receptors were involved. However, CD44 appears to play a key role in mast cells infiltration of BSM. In this context, it should be kept in mind that CD44 also mediates adhesion of lymphocytes to BSM (21). Indeed, using human T lymphocytes and BSM cells *in vitro*, Lazaar et al. (21) demonstrated that CD44 is necessary to VLA-4- and LFA-1-mediated cell–cell adhesion.

By contrast, we found in the present study that human mast cells adhere to BSM cells mainly through CD44 and pericellular matrix components.

In lung inflammation, however, the role of CD44 remains controversial (40). On the one hand, several studies have demonstrated that CD44 is a proinflammatory agent in a variety of murine models such as eosinophilic and T helper type 2 lung inflammation (17, 18). On the other hand, CD44^{-/-} mice develop exaggerated lung inflammatory responses including increased leukocyte infiltration and chemokine expression (41). However, there is no data regarding mast cell localization within the BSM in CD44^{-/-} mice (41). One possible explanation for such a discrepancy may be the association of different immunological phenotypes represented by CD44 variant isoforms which are predominantly expressed on activated leukocytes (22). In both mice and humans, CD44 gene can produce higher molecular mass CD44 isoforms by alternative splicing. These isoforms contain additional peptide sequences within the extracellular domain of CD44. Several studies focused on specific functions for some of these CD44 variant isoforms by using either blocking antibodies (24, 42) or generating specific knock-out mice (43). For example, early thymocyte development requires the expression of CD44 variant isoforms (42), and blocking of variants 6 and 7 prevents delayed-type hypersensitivity (24). Disruption of CD44v6v7 also prevents experimental colitis by increasing cell death in the inflammatory lesions (43), whereas CD44v6 can interfere with CD95 trimerization and hence block apoptosis signaling (44). In the present study performed in humans, we observed an increased expression of CD44v6 in both BSM cells from asthmatic patients when compared with healthy controls and mast cells after TGF- β 1 stimulation. Further studies should analyze the *in vivo* functions of CD44v isoforms in murine models of experimental asthma.

In conclusion, we have demonstrated the key role of CD51 and CD44v6 in mast cell adhesion to BSM in asthma via the extracellular matrix. This mechanism may account for the

very specific mast cell infiltration observed in asthma (2, 4). As it is believed that such infiltration contribute to long-term effect leading to bronchial hyperreactivity and remodeling, CD44v6 may thus represent a potentially attractive new target for asthma therapy.

We thank the staff of 'Service de Chirurgie Thoracique – CHU de Bordeaux' for the supply of human lung tissue. We thank Ursula Gunthert from Basel Switzerland for the supply of various antibodies against CD44 variants. We also thank Nadia El Bachachi and Vincent Pitard for their assistance and Derrick Robinson for his expertise in electron microscopy.

Funding

This study was supported by a grant from the Fondation pour la Recherche Médicale, France; the Société Française d'Allergologie et d'Immunologie Clinique, France and the Agence Nationale de la Recherche, France. The authors have no relationships to declare.

Supporting Information

Additional supporting information may be found in the online version of this article.

Methods and References.

Table S1. Effect of blocking Abs directed against cell adhesion molecules on mast cell-BSM cell adhesion.

Figure S1. Bronchial smooth muscle (BSM) cell purity.

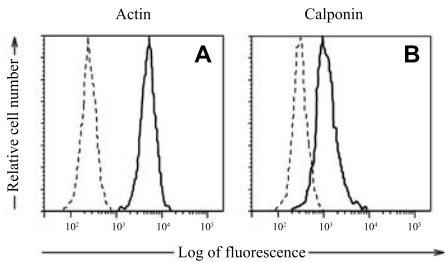
Figure S2. Human lung mast cell (HLMC) purity.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

1. Broide D. New perspectives on mechanisms underlying chronic allergic inflammation and asthma in 2007. *J Allergy Clin Immunol* 2008;**122**:475–480.
2. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. *N Engl J Med* 2002;**346**:1699–1705.
3. Carroll NG, Mutavdzic S, James AL. Distribution and degranulation of airway mast cells in normal and asthmatic subjects. *Eur Respir J* 2002;**19**:879–885.
4. Berger P, Girodet PO, Begueret H, Ousova O, Perng DW, Marthan R et al. Tryptase-stimulated human airway smooth muscle cells induce cytokine synthesis and mast cell chemotaxis. *FASEB J* 2003;**17**:2139–2141.
5. Brightling CE, Ammit AJ, Kaur D, Black JL, Wardlaw AJ, Hughes JM et al. The CXCL10/CXCR3 Axis Mediates Human Lung Mast Cell Migration to Asthmatic Airway Smooth Muscle. *Am J Respir Crit Care Med* 2005;**171**:1103–1108.
6. El-Shazly A, Berger P, Girodet PO, Ousova O, Fayon M, Vernejoux JM et al. Fractalkine produced by airway smooth muscle cells contributes to mast cell recruitment in asthma. *J Immunol* 2006;**176**:1860–1868.
7. Begueret H, Berger P, Vernejoux JM, Dubuisson L, Marthan R, Tunon-de-Lara JM. Inflammation of bronchial smooth muscle in allergic asthma. *Thorax* 2007;**62**:8–15.
8. Bradding P, Walls AF, Holgate ST. The role of the mast cell in the pathophysiology of asthma. *J Allergy Clin Immunol* 2006;**117**:1277–1284.
9. Thangam EB, Venkatesha RT, Zaidi AK, Jordan-Sciutto KL, Goncharov DA, Krymskaya VP et al. Airway smooth muscle cells enhance C3a-induced mast cell degranulation following cell-cell contact. *FASEB J* 2005;**19**:798–800.
10. Yang W, Kaur D, Okayama Y, Ito A, Wardlaw AJ, Brightling CE et al. Human lung mast cells adhere to human airway smooth muscle, in part, via tumor suppressor in lung cancer-1. *J Immunol* 2006;**176**:1238–1243.
11. Hollins F, Kaur D, Yang W, Cruse G, Saunders R, Sutcliffe A et al. Human airway smooth muscle promotes human lung mast cell survival, proliferation, and constitutive activation: cooperative roles for CADM1, stem cell factor, and IL-6. *J Immunol* 2008;**181**:2772–2780.
12. Thompson EE, Pan L, Ostrovskaya I, Weiss LA, Gern JE, Lemanske RF Jr et al. Integrin beta 3 genotype influences asthma and

- allergy phenotypes in the first 6 years of life. *J Allergy Clin Immunol* 2007;**119**:1423–1429.
13. Agis H, Fureder W, Bankl HC, Kundi M, Sperr WR, Willheim M et al. Comparative immunophenotypic analysis of human mast cells, blood basophil and monocytes. *Immunology* 1996;**87**:535–543.
 14. Columbo M, Bochner BS. Human skin mast cells adhere to vitronectin via the α 5 β 1 integrin receptor (CD51/CD61). *J Allergy Clin Immunol* 2001;**107**:554.
 15. Thompson RD, Wakelin MW, Larbi KY, Dewar A, Asimakopoulos G, Horton MA et al. Divergent effects of platelet-endothelial cell adhesion molecule-1 and β 3 integrin blockade on leukocyte transmigration in vivo. *J Immunol* 2000;**165**:426–434.
 16. Lackie PM, Baker JE, Gunthert U, Holgate ST. Expression of CD44 isoforms is increased in the airway epithelium of asthmatic subjects. *Am J Respir Cell Mol Biol* 1997;**16**:14–22.
 17. Katoh S, Matsumoto N, Kawakita K, Tomimaga A, Kincade PW, Matsukura S. A role for CD44 in an antigen-induced murine model of pulmonary eosinophilia. *J Clin Invest* 2003;**111**:1563–1570.
 18. Katoh S, Ishii N, Nobumoto A, Takeshita K, Dai SY, Shinonaga R et al. Galectin-9 Inhibits CD44-hyaluronan Interaction and Suppresses a Murine Model of Allergic Asthma. *Am J Respir Crit Care Med* 2007;**176**:27–35.
 19. Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B. CD44 is the principal cell surface receptor for hyaluronate. *Cell* 1990;**61**:1303–1313.
 20. Mackay CR, Terpe HJ, Stauder R, Marston WL, Stark H, Gunthert U. Expression and modulation of CD44 variant isoforms in humans. *J Cell Biol* 1994;**2**:71–82.
 21. Lazaar AL, Albelda SM, Pilewski JM, Brennan B, Pure E, Panettieri RA Jr. T lymphocytes adhere to airway smooth muscle cells via integrins and CD44 and induce smooth muscle cell DNA synthesis. *J Exp Med* 1994;**180**:807–816.
 22. Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol* 2003;**4**:33–45.
 23. Droll A, Dougherty ST, Chiu RK, Dirks JF, McBride WH, Cooper DL et al. Adhesive interactions between alternatively spliced CD44 isoforms. *J Biol Chem* 1995;**270**:11567–11573.
 24. Wittig B, Seiter S, Foger N, Schwarzler C, Gunthert U, Zoller M. Functional activity of murine CD44 variant isoforms in allergic and delayed type hypersensitivity. *Immunol Lett* 1997;**3**:217–223.
 25. Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA). 2008. Available from: <http://www.ginasthma.org>.
 26. Trian T, Benard G, Begueret H, Rossignol R, Girodet PO, Ghosh D et al. Bronchial smooth muscle remodeling involves calcium-dependent enhanced mitochondrial biogenesis in asthma. *J Exp Med* 2007;**204**:3173–3181.
 27. Berger P, Tunon-De-Lara JM, Savineau JP, Marthan R. Selected contribution: tryptase-induced PAR-2-mediated Ca^{2+} signaling in human airway smooth muscle cells. *J Appl Physiol* 2001;**91**:995–1003.
 28. Mark Duffy S, Berger P, Cruse G, Yang W, Bolton SJ, Bradding P. The K^{+} channel iKCA1 potentiates Ca^{2+} influx and degranulation in human lung mast cells. *J Allergy Clin Immunol* 2004;**114**:66–72.
 29. Brightling CE, Kaur D, Berger P, Morgan AJ, Wardlaw AJ, Bradding P. Differential expression of CCR3 and CXCR3 by human lung and bone marrow-derived mast cells: implications for tissue mast cell migration. *J Leukoc Biol* 2005;**77**:759–766.
 30. Butterfield JH, Weiler D, Dewald G, Gleich GJ. Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leuk Res* 1988;**12**:345–355.
 31. Trian T, Girodet PO, Ousova O, Marthan R, Tunon-de-Lara JM, Berger P. RNA interference decreases PAR-2 expression and function in human airway smooth muscle cells. *Am J Respir Cell Mol Biol* 2006;**34**:49–55.
 32. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;**3**: research0034.1–0034.11.
 33. Kruger-Krasagakes S, Grutzkau A, Krasagakis K, Hoffmann S, Henz BM. Adhesion of human mast cells to extracellular matrix provides a co-stimulatory signal for cytokine production. *Immunology* 1999;**98**:253–257.
 34. Berger P, Compton SJ, Molimard M, Walls AF, N'Guyen C, Marthan R et al. Mast cell tryptase as a mediator of hyperresponsiveness in human isolated bronchi. *Clin Exp Allergy* 1999;**29**:804–812.
 35. Berger P, Perng DW, Thabrew H, Compton SJ, Cairns JA, McEuen AR et al. Tryptase and agonists of PAR-2 induce the proliferation of human airway smooth muscle cells. *J Appl Physiol* 2001;**91**:1372–1379.
 36. Johnson PR, Roth M, Tamm M, Hughes M, Ge Q, King G et al. Airway smooth muscle cell proliferation is increased in asthma. *Am J Respir Crit Care Med* 2001;**164**:474–477.
 37. Roth M, Johnson PR, Borger P, Bihl MP, Rudiger JJ, King GG et al. Dysfunctional interaction of C/EBP α and the glucocorticoid receptor in asthmatic bronchial smooth-muscle cells. *N Engl J Med* 2004;**351**:560–574.
 38. Jalkanen S, Jalkanen M. Lymphocyte CD44 binds the COOH-terminal heparin-binding domain of fibronectin. *J Cell Biol* 1992;**116**:817–825.
 39. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 1992;**69**:11–25.
 40. Rothenberg ME. CD44—a sticky target for asthma. *J Clin Invest* 2003;**111**:1460–1462.
 41. Teder P, Vandivier RW, Jiang D, Liang J, Cohn L, Pure E et al. Resolution of lung inflammation by CD44. *Science* 2002;**296**:155–158.
 42. Schwarzler C, Oliferenko S, Gunthert U. Variant isoforms of CD44 are required in early thymocyte development. *Eur J Immunol* 2001;**31**:2997–3005.
 43. Wittig BM, Johansson B, Zoller M, Schwarzler C, Gunthert U. Abrogation of experimental colitis correlates with increased apoptosis in mice deficient for CD44 variant exon 7 (CD44v7). *J Exp Med* 2000;**191**:2053–2064.
 44. Mielgo A, van Driel M, Bloem A, Landmann L, Gunthert U. A novel antiapoptotic mechanism based on interference of Fas signaling by CD44 variant isoforms. *Cell Death Differ* 2006;**13**:465–477.



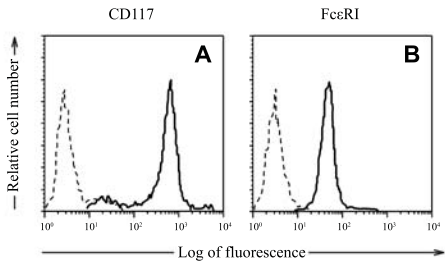


Figure legends

Figure S1. Bronchial smooth muscle (BSM) cell purity.

FACS representative histograms were obtained using non asthmatic BSM cells. Alpha-smooth muscle actin (**A**) or calponin (**B**) expression was assessed using irrelevant Ab (dotted lines) or specific Abs (full lines). BSM cells highly expressed actin (% of positive cells: 98.0) and calponin (% of positive cells: 87.9).

Figure S2. Human lung mast cell (HLMC) purity.

FACS representative histograms were obtained using HLMC. CD117 (**A**) or FcεRI (**B**) expression was assessed using irrelevant Ab (dotted lines) or specific Abs (full lines). HLMC highly expressed CD117 (% of positive cells: 83.0) and FcεRI (% of positive cells: 95.3).

ONLINE REPOSITORY

Original Article

Mast cell adhesion to bronchial smooth muscle in asthma specifically depends on CD51 and CD44 variant 6

Pierre-Olivier Girodet, MD, PhD,^{a,b,c} Annaig Ozier, MD,^{a,b,d} Thomas Trian, PhD,^{a,b} Hugues Begueret, MD, PhD,^{a,b,d} Olga Ousova, PhD,^{a,b} Jean-Marc Vernejoux, MD,^d Pascal Chanez, MD, PhD,^e Roger Marthan, MD, PhD,^{a,b,d} Patrick Berger, MD, PhD,^{a,b,d} and J. Manuel Tunon de Lara, MD, PhD,^{a,b,d}

^aUniversité de Bordeaux, Bordeaux, France

^bINSERM U885, Bordeaux, France

^cCHU de Bordeaux-INSERM, Centre d'Investigation Clinique, Bordeaux, France

^dCHU de Bordeaux, Bordeaux, France

^eUniversité de la Méditerranée AP-HM, Marseille, France

Corresponding author

Pierre-Olivier Girodet, MD, PhD,
INSERM U885, Université de Bordeaux,
CHU de Bordeaux-INSERM, Centre d'Investigation Clinique,
146 rue Léo Saignat,
F-33076 Bordeaux cedex, France

Telephone: + 33 (0) 5 57 57 15 60

Fax: + 33 (0) 5 57 57 46 71

E-mail: pierre-olivier.girodet@pharmaco.u-bordeaux2.fr

METHODS

Study population and human tissue collection

A total of 11 patients with severe persistent asthma, and 35 non asthmatic controls were prospectively recruited from the “Centre Hospitalier Universitaire de Bordeaux”.

Asthmatic patients were enrolled using the following inclusion criteria. Patients >18 yr had to exhibit characteristic symptoms (i.e., wheezing and breathlessness), as well as bronchial hyperresponsiveness confirmed by either a significant improvement by >15% in the forced expiratory volume in 1 s (FEV₁) 10 min after the inhalation of 200 µg of salbutamol, or a provocative concentration of methacholine required to lower the FEV₁ by 20% (PC20) <4 mg/ml, according to the American Thoracic Society criteria (1). Patients had to be graded as severe persistent asthmatics according to the Global Initiative for Asthma guidelines based on both clinical features and daily medication regimen (2). All patients also had to be lifelong nonsmokers with no recent exacerbation (<3 mo). Exclusion criteria were recent bacterial or viral infections (<3 mo).

Control subjects were enrolled using the following inclusion criteria. Subjects >18 yr underwent a thoracotomy because of hemoptysis or abnormal image on x ray. They had to be asymptomatic without any treatment. Exclusion criteria were history of asthma or recent bacterial or viral infections (<3 mo).

All subjects gave their written informed consent to participate in the study, after the nature of the procedure had been fully explained. This study followed recommendations outlined in the Helsinki Declaration and received approval from the local ethics committee.

Bronchial specimens from all subjects were obtained by either fiberoptic bronchoscopy or lobectomy (3-5). Briefly, fiberoptic bronchoscopy was performed after the anesthesia of the upper airways was achieved with lidocaine 10% spray. The fiberoptic bronchoscope (FB-5V; Pentax) was introduced, and biopsies were taken from various bronchial carinae from the middle lobe. Specimens were immediately transferred to the laboratory in a sterile container containing Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Cergy Pontoise, France).

Cell culture and challenge

Bronchial smooth muscle (BSM) cells were cultured as described previously (5, 6). Briefly, collected tissues were carefully dissected under a dissecting microscope. Smooth muscle explants were cultured and maintained in DMEM containing 10% (v/v) FCS (Invitrogen).

Only cells passaged two to four times with trypsin-EDTA (Invitrogen) were used for this study.

Human lung mast cells (HLMC) were dispersed from macroscopically normal lung obtained within 1 h of resection for lung cancer (7, 8). The enzymatic dispersal procedure used both collagenase and hyaluronidase (both from Sigma-Aldrich, Saint Quentin-Fallavier, France) as described previously (9). Mast cells were then purified using immunomagnetic affinity selection with the anti-CD117-coated immunomagnetic beads (DynaL collection kit, Dynal, Oslo, Norway) (7, 8). Isolated HLMC were cultured in DMEM containing 10% (v/v) FCS, 100 ng/ml SCF, 50 ng/ml IL-6 and 10 ng/ml IL-10 (all from R&D Systems, Abingdon Oxon, UK) as described previously (7).

The human mast cell line HMC-1 (10) was also cultured in DMEM containing 10% (v/v) FCS.

Cell purity was assessed by both immunocytochemistry and flow cytometry (Beckton Dickinson, Pont de Claix, France) using anti-alpha smooth muscle actin (Sigma-Aldrich) and anti-calponin (Santa Cruz Biotechnology, Heidelberg, Germany) antibodies for BSM cells (Figure S1) or anti-CD117 (Dako, Trappes, France) and anti-FcεRI (Millipore, Saint-Quentin-en-Yvelines, France) antibodies for HLMC (Figure S2). No specific staining was found with anti-cytokeratin 18 (Sigma-Aldrich), anti-fibroblast surface protein (Sigma-Aldrich), anti-factor VIII (Dako, Trappes, France), or an irrelevant antibody (Sigma-Aldrich).

Adhesion molecules expression on mast cell and BSM cell was also assessed by flow cytometry using various antibodies. Cells were fixed with 4% paraformaldehyde (Thermo Fisher Scientific, Illkirch, France) and then washed twice and incubated for 30 min on ice with mouse Abs against human CD51 (Beckman Coulter, Roissy CDG, France), panCD44 (Dako) or CD44v4, v6 or v9 (11) or isotype control Abs. Cells were analyzed for their fluorescence intensity using FACSCalibur (Beckton Dickinson). Results are expressed in mean fluorescence intensity (MFI) and percentage of positive cells.

Mast cell-BSM cell adhesion

HMC-1 or HLMC adhesion to BSM cells was initially assessed using radio-labeled mast cells as adapted from Lazaar et al (12). Briefly, subconfluent mast cells were pulsed with 10 µCi/ml [³H]-thymidine (Amersham Biosciences, Orsay, France) for 16h at 37°C. After cell passage, [³H]-labeled mast cells were added to 96-well culture plates coated with 3% BSA, 100 µg/ml fibronectin (both from Sigma-Aldrich) or growth-arrested BSM cell monolayer and allowed to adhere for another 1 h at 37°C. Non adherent cells were removed by washing.

In additional triplicate wells, cells were not washed in order to assess the total input counts. [³H]-labeled cells were harvested on a glassfibre filter (Filtermat A, GF/C, PerkinElmer, Courtaboeuf, France) and counted in scintillant on a 2000 CA TriCarb liquid scintillation analyzer (1450 microbeta, Wallac, Trilux, PerkinElmer, CT). Percentage of maximal adhesion was calculated from counts recovered from adherent cells as compared to that of total cells.

In another set of experiments, BSM cells and/or mast cells were pre-incubated before adhesion assay with hyaluronidase, collagenase (0.1-1 µg/ml for 1 h, both from Sigma-Aldrich) or various blocking Abs (10 µg/ml for 45 min) against human adhesion molecules expressed on either BSM cell or mast cell (12-16). These Abs included murine monoclonal Abs against CD44 (Dako, Trappes, France), CD51, intercellular adhesion molecule 1 (ICAM-1, CD54), vascular cell adhesion molecule 1 (VCAM-1, CD106) (all from Beckman Coulter), membrane fractalkine and stem cell factor (SCF) (both from R&D Systems), or isotype controls.

In another set of experiments, BSM cells (10 000 cells/well) were cultured in 8-well chamber slides (Invitrogen) or cell culture inserts (polyethylene terephthalate, VWR International, Strasbourg, France), and grown until confluency at 37°C. After a 3 days serum deprivation, BSM cells were not stimulated or stimulated with 100 ng/ml TNF-α for 72 h. Mast cells were challenged with 0 or 1 ng/ml TGF-β1 for 1 h and then added to the BSM cells monolayer for 1 h at 37°C. After washing 5 times with PBS (Sigma-Aldrich) to remove non-adherent cells, slides were then processed for either confocal microscopy as described previously (4) or electron microscopy.

For confocal microscopy, cells were fixed with cold methanol (VWR international) for 20 min on ice. After blocking of non-specific staining with 3% BSA (Sigma-Aldrich), mast cells were stained with a mouse anti-human tryptase Ab (Dako) and Texas Red[®]-conjugated goat anti-mouse Ab (Molecular Probes, Eugene, OR). BSM cells were then stained with a FITC-conjugated mouse anti-human α-smooth muscle actin Ab (Sigma-Aldrich). After washing, the slides were mounted with Fluorescent mounting medium (Dako). Confocal differential-interference-contrast images were obtained using Fluoview laser scanning microscope (Nikon, Paris, France) and x60 oil-immersion objective. Z-series sections were recorded in successive z-axis serial sections at 0.5-µm intervals and were composed of optical sections in the x-y optical plane. Sections were reconstituted in 3D images using Imaris Software (Bitplane, Zürich, Switzerland).

For electron microscopy, cells were fixed with 2.5% glutaraldehyde at 4°C in 0.1 M sodium cacodylate buffer (both from Euromedex, Souffelweyersheim, France) for 2 h. Samples were

post-fixed in 1% osmium tetroxide in sodium cacodylate buffer for 90 min, dehydrated through graded series of ethanol (70-100%) and embedded in epoxy resin (Araldite, Biorad, Cambridge, MA). Ultra-thin sections were then cut, mounted with 100-mesh thin-bar grids and stained with uranyl acetate and lead citrate. Electronic images were obtained using a Philips 301 electron microscope (Philips Electron Optics, Eindhoven, The Nederland).

RNA extraction, reverse transcription, and real-time quantitative PCR

Total RNA was prepared from stimulated or nonstimulated BSM cells and mast cells using Trizol[®] (Invitrogen) and mRNA was reverse transcribed using 1 µg total RNA, oligo d(T) and AMV reverse transcriptase (Promega, Charbonnières, France), as previously described (17).

Real time quantitative PCR was performed on a Rotor-Gene[™] 2000 (Corbett Research, Mortlake, Sydney, Australia). Briefly, triplicate PCR reactions were assembled in 0.1 ml strip tubes containing cDNA from 10 ng of total RNA, 0.2 µl 50x Titanium Taq DNA Polymerase, 1x Titanium Taq PCR Buffer (Clontech Laboratories, Ozyme, Saint Quentin en Yvelines, France), 1 mM dNTP, 100 mM each of the appropriate primer, 0.5x Sybr Green I (Invitrogen). The PCR was performed under the following conditions: denaturation at 95°C for 15 s, annealing temperature (64-70°C) depending on specific primers for 15 s and extension at 70°C for 30 s for 30 cycles. Data collection was performed after each extension step, at a temperature at least 3.5°C lower than the melting temperature of the amplicon (generally between 79-85°C) to eliminate non-specific fluorescence signals. PCR negative controls were systematically made using water instead of cDNA or RNA sample without the reverse transcriptase step. All specific primers were designed using the primer analysis software Oligo[®] 6.6 (Molecular Biology Insights, Cascade, CO) and were ordered from Sigma-Aldrich. Primers, sense and anti-sense, were as follows: for human CD51 (NM_002210), forward 5'-CTCGCCAGGTGGTATGT-3' and reverse 5'-TGAGATACA ACTGGGCTTACT-3'; for human CD44 (NM_000610), forward 5'-GCTGATCATCTTGGCATCCCT-3' and reverse 5'-GCTGGCCTCTCCGTTGAGT-3'; for human collagen type I α1 (NM_000089), forward 5'-GACTGGTGAGACCTGCGTGTA-3' and reverse 5'-GGCCGCCATACTCGAACT-3'; for tyrosine-3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) (NM_003406), forward 5'-GGGGATGTGGAATTTTTATACAAG-3' and reverse 5'-CGCCAGGACAAACCAGTATGTAG-3'; for hypoxanthine phosphoribosyltransferase 1 (HPRT-1) (NM_000194), forward 5'-TGTTGGATATGCCCTTGACTA-3' and reverse 5'-AGATGGCCACAGGACTAGAAC-3'; for human acidic ribosomal phosphoproteins (PO)

(NM_001002), forward 5'-CAACGGGTACAAACGAGTC-3' and reverse 5'-CTTCCTTGGCTTCAACCTTAG-3'. The average efficiency of the PCR reactions was always more than 90%. The specificity of the amplified PCR products was examined with the melting curve analysis and also in 2% agarose gel containing ethidium bromide. The RT-PCR expression of human CD44, CD51 and collagen type I α 1 was thus presented as an arbitrary unit and normalized to endogenous references (geometric averaging of the internal house keeping genes i.e. YWHAZ, HPRT-1 and PO) according to geNorm software (18).

Immunoblotting

Whole lysates from asthmatic or non asthmatic BSM cells and mast cells were collected using 1% Triton X-100 lysis buffer in the presence of 2mM Na orthovanadate, 1 mM EDTA, 50 μ g/mL aprotinin, 100 μ M leupeptin, 1 mM 1,4 Dithio-DL-Treitol, and 1 mM amino-ethyl-benzenesulfonyl fluoride hydrochloride (all from Sigma-Aldrich). Cellular extracts were reduced with β -mercaptoethanol, subjected to electrophoresis on a 10% acrylamide reducing gel, and transferred to polyvinylidene fluoride membranes (Immobilon-PTM Transfer Membrane, Millipore, Billerica, MA). The immunoblots were then developed using monoclonal mouse anti-human collagen I α (Santa Cruz) and monoclonal mouse anti-human β -actin (Sigma-Aldrich) for 14 h at 4°C. For amplification, biotinylated goat secondary anti-mouse IgG (Bio-Rad Laboratories, Marnes-la-Coquette, France) for 90 min at room temperature and a streptavidin-biotinylated horseradish peroxidase complex (Dako) were used. Immunoblots were revealed by enhanced chemiluminescence (Uptima, Interchim, Montluçon, France). Blot images were acquired using BioCaptMW software (Thermo Fisher Scientific). Band densities were quantified using ImageJ software and normalized to that of β -actin.

Statistical analysis

Values are presented as the mean $\pm$ the SEM. Statistical analysis was performed using NCSS 2001 software. Statistical significance was analyzed by ANOVA, Kruskal-Wallis and paired Student's *t* test. P value <0.05 was taken as the threshold for statistical significance.

References

1. Crapo RO, Casaburi R, Coates AL, Enright PL, Hankinson JL, Irvin CG, et al. Guidelines for methacholine and exercise challenge testing-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. *Am J Respir Crit Care Med*. 2000 Jan;161(1):309-29.
2. Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA). 2008; Available from: <http://www.ginasthma.org>.
3. Berger P, Girodet PO, Begueret H, Ousova O, Perng DW, Marthan R, et al. Tryptase-stimulated human airway smooth muscle cells induce cytokine synthesis and mast cell chemotaxis. *Faseb J*. 2003 Nov;17(14):2139-41.
4. El-Shazly A, Berger P, Girodet PO, Ousova O, Fayon M, Vernejoux JM, et al. Fractalkine produced by airway smooth muscle cells contributes to mast cell recruitment in asthma. *J Immunol*. 2006 Feb 1;176(3):1860-8.
5. Trian T, Benard G, Begueret H, Rossignol R, Girodet PO, Ghosh D, et al. Bronchial smooth muscle remodeling involves calcium-dependent enhanced mitochondrial biogenesis in asthma. *J Exp Med*. 2007 Dec 24;204(13):3173-81.
6. Berger P, Tunon-De-Lara JM, Savineau JP, Marthan R. Selected contribution: tryptase-induced PAR-2-mediated Ca(2+) signaling in human airway smooth muscle cells. *J Appl Physiol*. 2001 Aug;91(2):995-1003.
7. Mark Duffy S, Berger P, Cruse G, Yang W, Bolton SJ, Bradding P. The K⁺ channel iKCA1 potentiates Ca²⁺ influx and degranulation in human lung mast cells. *J Allergy Clin Immunol*. 2004 Jul;114(1):66-72.
8. Brightling CE, Kaur D, Berger P, Morgan AJ, Wardlaw AJ, Bradding P. Differential expression of CCR3 and CXCR3 by human lung and bone marrow-derived mast cells: implications for tissue mast cell migration. *J Leukoc Biol*. 2005 May;77(5):759-66.
9. Okayama Y, Hunt TC, Kassel O, Ashman LK, Church MK. Assessment of the anti-c-kit monoclonal antibody YB5.B8 in affinity magnetic enrichment of human lung mast cells. *J Immunol Methods*. 1994 Mar 10;169(2):153-61.
10. Butterfield JH, Weiler D, Dewald G, Gleich GJ. Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leuk Res*. 1988;12(4):345-55.
11. Mackay CR, Terpe HJ, Stauder R, Marston WL, Stark H, Gunthert U. Expression and modulation of CD44 variant isoforms in humans. *J Cell Biol*. 1994 Jan;124(1-2):71-82.

12. Lazaar AL, Albelda SM, Pilewski JM, Brennan B, Pure E, Panettieri RA, Jr. T lymphocytes adhere to airway smooth muscle cells via integrins and CD44 and induce smooth muscle cell DNA synthesis. *J Exp Med.* 1994;180(3):807-16.
13. Agis H, Fureder W, Bankl HC, Kundi M, Sperr WR, Willheim M, et al. Comparative immunophenotypic analysis of human mast cells, blood basophil and monocytes. *Immunology.* 1996;87:535-43.
14. Lazaar AL, Amrani Y, Hsu J, Panettieri RA, Jr., Fanslow WC, Albelda SM, et al. CD40-mediated signal transduction in human airway smooth muscle. *J Immunol.* 1998;161(6):3120-7.
15. Hakonarson H, Kim C, Whelan R, Campbell D, Grunstein MM. Bi-directional activation between human airway smooth muscle cells and T lymphocytes: role in induction of altered airway responsiveness. *J Immunol.* 2001;166(1):293-303.
16. Freyer AM, Johnson SR, Hall IP. Effects of growth factors and extracellular matrix on survival of human airway smooth muscle cells. *Am J Respir Cell Mol Biol.* 2001;25(5):569-76.
17. Trian T, Girodet PO, Ousova O, Marthan R, Tunon-de-Lara JM, Berger P. RNA interference decreases PAR-2 expression and function in human airway smooth muscle cells. *Am J Respir Cell Mol Biol.* 2006 Jan;34(1):49-55.
18. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 2002 Jun 18;3(7):research0034.1-11.

Table S1. Effect of blocking Abs directed against cell adhesion molecules on mast cell-BSM cell adhesion

	Mast cell	Nonstimulated	TGF- β 1 (1 ng/ml – 1 h)
	BSM cell	Nonstimulated	TNF- α (100 ng/ml – 72 h)
Irrelevant / BSM cell		32.7 $\pm$ 3.9	47.2 $\pm$ 5.9*
Anti-ICAM-1 / BSM cell		41.2 $\pm$ 4.1	50.9 $\pm$ 8.9*
Anti-VCAM-1 / BSM cell		37.6 $\pm$ 5.3	54.6 $\pm$ 7.4*
Anti-CD40L / BSM cell		33.6 $\pm$ 3.5	46.1 $\pm$ 3.9*
Anti-CD117 / BSM cell		33.1 $\pm$ 3.6	45.7 $\pm$ 6.9*
Anti-fractalkine / BSM cell		31.6 $\pm$ 2.5	48.0 $\pm$ 6.1*
Anti-ICAM-1 / Mast cell		35.9 $\pm$ 6.0	45.2 $\pm$ 2.3*

Effect of blocking Abs on mast cells-BSM cells adhesion. Values are means $\pm$ SEM of 6 independent experiments. *, $p < 0.05$ versus nonstimulated cells condition. No significant difference was found when each experimental condition with blocking Ab was compared to the corresponding condition with irrelevant Ab.

Figure legends

Figure S1. Bronchial smooth muscle (BSM) cell purity.

FACS representative histograms were obtained using non asthmatic BSM cells. Alpha-smooth muscle actin (**A**) or calponin (**B**) expression was assessed using irrelevant Ab (dotted lines) or specific Abs (full lines). BSM cells highly expressed actin (% of positive cells: 98.0) and calponin (% of positive cells: 87.9).

Figure S2. Human lung mast cell (HLMC) purity.

FACS representative histograms were obtained using HLMC. CD117 (**A**) or FcεRI (**B**) expression was assessed using irrelevant Ab (dotted lines) or specific Abs (full lines). HLMC highly expressed CD117 (% of positive cells: 83.0) and FcεRI (% of positive cells: 95.3).