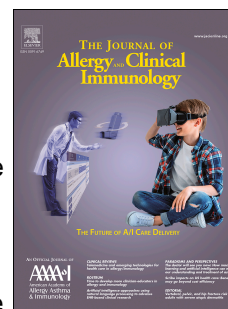


Journal Pre-proof

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PII: S0091-6749(22)00148-8

DOI: <https://doi.org/10.1016/j.jaci.2022.01.022>

Reference: YMAI 15456

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 27 September 2021

Revised Date: 17 January 2022

Accepted Date: 21 January 2022

Please cite this article as: Celle A, Esteves P, Cardouat G, Beaufils F, Eyraud E, Dupin I, Maurat E, Lacomme S, Ousova O, Begueret H, Thumerel M, Marthan R, Girodet P-O, Berger P, Trian T, Rhinovirus infection of bronchial epithelium induces specific bronchial smooth muscle cell migration of severe asthmatic patients, *Journal of Allergy and Clinical Immunology* (2022), doi: <https://doi.org/10.1016/j.jaci.2022.01.022>.

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Rhinovirus infection of bronchial epithelium induces specific bronchial smooth muscle cell migration of severe asthmatic patients

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CONFLICT OF INTEREST DISCLOSURE STATEMENT

- P. Berger reports grant from AstraZeneca within the submitted work. P. Berger also reports grants and personal fees from Novartis; grants, personal fees and non-financial support from Boehringer Ingelheim; personal fees and non-financial support from Chiesi, AstraZeneca, and Sanofi; personal fees from Menarini, and TEVA, outside the submitted work; In addition, he has a delivered patent (EP N°15152886.6 *i.e.*, New compositions and methods of treating and/or preventing Chronic Obstructive Pulmonary Disease), a submitted patent (22605-FR, *i.e.*, Geometric characterization of airways using MRI) and a submitted patent (EP N°20173595.8 *i.e.*, New compositions and methods of treating COVID-19 Disease), all outside the submitted work.

- PO. Girodet reports research support from AstraZeneca outside the submitted work. In addition, he has a delivered patent (EP 15152886.6 *i.e.*, New compositions and methods of treating and/or preventing Chronic Obstructive Pulmonary Disease) and a submitted patent (EP N°20173595.8 *i.e.*, New compositions and methods of treating COVID-19 Disease), all outside the submitted work.

- I. Dupin has a delivered patent (EP 15152886 *i.e.*, New compositions and methods of treating and/or preventing Chronic Obstructive Pulmonary Disease), and a submitted patent (EP N°20173595.8 *i.e.*, New compositions and methods of treating COVID-19 Disease), all outside the submitted work.

- F. Beaufils reports personal fees and non-financial supports from Novartis, Chiesi and AstraZeneca outside the submitted work.

- All other authors have no conflict of interest

47 **Sources of funding**

48 The “Agence Nationale pour la Recherche” (ANR, ROSAE Project CE14-0015-01). The
49 “Fondation de l’Université de Bordeaux” (“Fonds FGLMR/AVAD pour les maladies chroniques”)
50 and an unrestricted grant from AstraZeneca.

51

ABSTRACT

Background: Patients with severe asthma show an increase in both exacerbation frequency and bronchial smooth muscle (BSM) mass. Rhinovirus (RV) infection of the bronchial epithelium (BE) is the main trigger of asthma exacerbations. Histological analysis of biopsies shows that a close connection between BE and hypertrophic BSM is a criterion for severity of asthma.

Objective: We hypothesized that RV infection of BE specifically increases asthmatic BSM cell migration.

Methods: Serum samples, biopsies or BSM cells were obtained from 86 patients with severe asthma and 31 non-asthmatic subjects. BE cells from non-asthmatic subjects were cultured in an air-liquid interface and exposed to RV-16. Migration of BSM cells was assessed in response to BE supernatant using chemotaxis assays. Chemokine concentrations were analyzed by transcriptomics and ELISAs. Immunocytochemistry, western blotting and flow cytometry were used to quantify CXCR3 isoform distribution. CXCR3 downstream signaling pathways were assessed by calcium imaging and western blots.

Results: BSM cells from severe asthmatic patients specifically migrated toward RV-infected BE, whereas those from non-asthmatic subjects did not. This specific migration is driven by BE CXCL10, which was increased *in vitro* in response to RV infection as well as *in vivo* in serum from exacerbating patients with severe asthma. The mechanism is related to both decreased expression and activation of the CXCR3-B-specific isoform in severe asthmatic BSM cells.

Conclusion: We have demonstrated a novel mechanism of BSM remodeling in severe asthmatic patients following RV exacerbation. This study highlights the CXCL10/CXCR3-A axis as a potential therapeutic target in severe asthma.

Key message:

- CXCL10 induces specific migration of severe asthmatic BSM cells only.
- Such a specific migration is related to a decreased expression and activation of the CXCR3-B-specific isoform in severe asthmatic BSM cells

Capsule summary: This study demonstrates that CXCL10/CXCR3-A axis is specifically involved in bronchial smooth muscle remodeling in patients with severe asthma following RV infection and could be a new therapeutic target, particularly in patients with frequent exacerbations.

Key-words: Asthma, Bronchial smooth muscle, Exacerbation, Rhinovirus

Abbreviations:

BSM: Bronchial smooth muscle

BE: Bronchial epithelium

RV: Rhinovirus

PDGF: Platelet-derived growth factor

- 91 GINA: Global Initiative for Asthma
- 92 MOI: Multiplicity of infection
- 93 DMEM: Dulbecco modified Eagle's minimal essential medium
- 94 FBS: Fetal bovine serum
- 95 PBMC: Peripheral blood mononuclear cells
- 96 MFI: median fluorescence intensity
- 97 BMI: Body mass index
- 98 FEV1: Forced expiratory volume in 1 second
- 99 FVC: forced vital capacity
- 100 PKA: Protein kinase A
- 101 PKC : Protein kinase C

INTRODUCTION

Asthma is a frequent although heterogeneous airway disease that results in a major economic burden¹. Asthma pathophysiology involves chronic inflammation of both large and small airways, as well as bronchial remodeling. The latter is characterized by various structural changes in the bronchial wall, including epithelial alteration, increased subepithelial membrane thickness, mucous gland hypertrophy, neoangiogenesis and increased bronchial smooth muscle (BSM) mass². The most crucial feature of bronchial remodeling appears to be BSM remodeling, since increased BSM mass is associated with both impaired lung function^{3, 4} and a higher exacerbation rate⁵. These features have poor prognostic value in asthma and characterize most severe asthmatic patients¹. The link between asthma exacerbations and BSM remodeling has been clinically demonstrated by the effects of bronchial thermoplasty, which reduces both BSM mass⁶ and exacerbation rate after 3 months for up to 5 years of follow-up in severe asthmatic patients^{7, 8}. Moreover, we previously showed that the calcium channel inhibitor gallopamil administered for 12 months in severe asthmatic patients reduced both BSM thickness and, subsequently, the exacerbation rate compared to placebo in a proof-of-concept clinical trial⁹. However, the mechanism underlying the relationship between asthma exacerbations and BSM remodeling remains unknown.

The pathophysiology of asthmatic BSM remodeling is complex and is associated with various cellular dysfunctions including increased BSM cell proliferation and migration². Whereas increased asthmatic BSM cell proliferation has been, reproducibly, demonstrated *in vitro*^{10, 11}, its contribution *in vivo* remains a matter of debate^{4, 12}. Moreover, non-organized contractile elements have been identified outside BSM bundles⁴, particularly in severe asthma, suggesting either the

recruitment of circulating hematopoietic progenitor cells (*e.g.*, fibrocytes)^{13, 14} and/or an increased migration of asthmatic BSM cells. Such BSM cell migration has been demonstrated *in vitro* in response to leukotrienes¹⁵, CCL19¹⁶, YKL-40¹⁷, CXCL2 and CXCL3¹⁸. The migration of BSM cells induced by YKL-40¹⁷, CXCL2 or CXCL3¹⁸ was more pronounced when BSM cells originated from asthmatic patients. However, data regarding asthmatic BSM cell migration remain scarce, although they would be helpful for identifying new therapeutic strategies.

Viral infection of the bronchial epithelium (BE) has been implicated in 80% of asthma exacerbations in children and adolescents and in 60% of those in adults¹⁹. Human rhinovirus (RV) is the dominant viral pathogen, and RV causes 60% of all virus-induced asthma exacerbations¹⁹. At the cellular level, RV-infected basal undifferentiated BE cells produce more CCL5, CXCL8, and CXCL10, and induce the migration of non-asthmatic BSM cells²⁰. However, whether or not these chemokines represent innovative targets to treat severe asthma remodeling remains unknown.

The aims of the present study were thus: (i) to analyze the effect of RV infection of a differentiated BE, reconstituted *in vitro*, on the migration of BSM cells obtained from fully characterized severe asthmatic patients and non-asthmatic subjects; and (ii) to decipher the mechanisms of such *in vitro* migration. Finally, to evaluate the potential therapeutic relevance of the above mentioned *in vitro* findings, we analyzed biological samples of asthmatic patients according to their clinical status.

METHODS

See “Online Repository materials” for extensive methods

Study population

Non-asthmatic subjects and patients with severe asthma were prospectively recruited at the university hospital of Bordeaux, from the “COBRA” cohort, according to ATS/ERS criteria and GINA recommendations ^{1, 21}. All patients gave their written informed consent to participate to the study.

Immunohistochemistry

Assessable BSM was identified by a pathologist in a blinded fashion by using both morphologic characteristics and α -smooth muscle actin (α -SMA) staining, as previously described ^{4, 12}. We analyzed the distance (in μ m) between the BE basement membrane and the inner limit of the BSM at regular intervals of 50 μ m, as previously described ^{22, 23}.

Cell culture

BSM and BE cell cultures were performed as previously described ²⁴. Differentiated BE was obtained in an air liquid interface on cell culture inserts (Corning, New York, NY) after 21 days of culture (see Figure S1 in the Online Repository). We used human rhinovirus RV-16, obtained from ATCC (LGC Standards, Molsheim, France), at a multiplicity of infection (MOI) of 0.1. Infection of BE by RV was performed during 1 hour in DMEM supplemented with 2% FBS at the apical side of the epithelium. The viral solution was then removed from the apical side and BE cells were cultured for 24h prior to the collection of the basal medium.

BSM cell migration

Real-time cell migration of BSM cells was measured using a μ -slide chemotaxis system from Ibidi (Martinsried, Germany), according to the manufacturer's instructions. For all experiments, BSM cells were allowed to attach to the chamber 6h prior to the migration assay. BSM attachment was assessed by immunocytochemistry using α -smooth muscle actin antibody (see Figure S2 in the Online Repository). In some experiments, BSM cells were exposed to CXCR3 blocking antibody (R&D Systems, Minneapolis, MN) prior to the migration assay. Phase contrast images were recorded for 24 h with a time lapse of 30 min (see Video 1 in the Online Repository).

Multiplex Gene Expression Analysis

mRNA from non-infected and infected BE cells was extracted (AllPrep® DNA/RNA/Protein Mini Kit, Qiagen, Hilden, Germany) and the expression level was assessed with nCounter® FLEX using the inflammation panel (nanoString, Seattle, WA), according to the manufacturer's instructions.

ELISA

Human CXCL10 and CXCL9 concentration were quantified using ELISA following manufacturer's recommendations (R&D Systems).

Western blot

BSM cell total protein lysis was performed using RIPA lysis buffer (Sigma-Aldrich, Saint-Louis, MO). Total cellular extracts were loaded onto a 4-20% SDS-PAGE gel (Bio-Rad, Hercules, CA), transferred onto nitrocellulose membranes and visualized with antibodies directed against pan-

CXCR3, CXCR3-B, PKC (T514) and PKA (all from R&D Systems). In additional experiments, total protein lysate was extracted from microscopically dissected BSM tissues obtained from bronchial biopsies or bronchial surgical specimens (Sigma-Aldrich).

CD3⁺ T cell purification

Peripheral blood mononuclear cells (PBMCs) were separated from the whole blood by Ficoll-Hypaque (Eurobio Scientific, Les Ulis, France) density gradient centrifugation. CD3⁺ T cells were purified by positive selection using CD3 microbeads associated with LS columns (Miltenyi Biotech, Paris, France).

Flow cytometry

Membrane expression of both pan-CXCR3 and CXCR3-B (both from R&D Systems) was assessed in BSM and CD3⁺ T cells by flow cytometry (BD FACSCanto II, BD Biosciences, Franklin Lakes, NJ). The results are presented as a ratio of the median fluorescence intensity (MFI) normalized to the MFI of the IgG isotype control (R&D systems).

Immunocytochemistry of BSM cells

BSM cells were stained with antibodies against α -smooth muscle actin, calponin (both from Sigma-Aldrich), pan-CXCR3 and CXCR3-B (both from R&D Systems). Nuclei were stained with DAPI. Slides were imaged with confocal microscopy (Zeiss, Oberkochen, Germany).

Real-time Ca²⁺ fluorescence confocal imaging

BSM cells were incubated with the $[Ca^{2+}]$ probe fluo-4/AM (Invitrogen, Cergy Pontoise, France). Fluorescence was observed under a Nikon D-Eclipse C1 confocal scanning microscope. A calcium response was considered positive when $\Delta(F/F_0)$ was > 0.1 .

Statistical analyses

Statistical analyses were performed with Prism 6 software (GraphPad, San Diego, CA). Statistical significance, defined as $P < 0.05$, was analyzed by the dedicated tests mentioned in the figure legends.

RESULTS

Study populations

For *in vitro* experiments, we recruited a total of 20 non-asthmatic subjects and 13 severe asthmatic patients, whose characteristics are presented in Table 1. Both populations were similar in terms of age, sex and BMI. Not surprisingly, the severe asthmatic patients had significantly lower FEV1 values and FEV1/FVC ratio than the non-asthmatic subjects. Likewise, the severe asthmatic patients were also significantly different from the non-asthmatic subjects in terms of therapeutic pressure (Table 1).

For *ex vivo* experiments, we recruited two independent sets of patients with either biopsies or serum. We analyzed 11 non-asthmatic and 22 asthmatic patients for histological measurements and 51 severe asthmatic patients for serum concentration analyses. Both patients' characteristics are presented in the Online Repository tables **S1** and **S2**, respectively.

For these 3 types of analyses, we paid a special attention to recruit asthmatic patients with similar clinical and functional characteristics. Indeed, there was no significant difference in either age, BMI, FEV1 or FEV1/FVC ratio in the 3 groups of patients using one-way ANOVA (Data not shown)

BSM is closer to BE in the severe asthmatic patients

We first measured the distance between BSM and BE basement membrane in bronchial biopsies obtained from 11 non-asthmatic subjects and 22 severe asthmatic patients, in a blinded fashion regarding the clinical status of the biopsies (see Table S1 in the Online Repository). We found that the distance between BSM and BE basement membrane was significantly lower in the severe asthmatic patients than in the non-asthmatic subjects (Figure 1A, B and C).

RV-infected BE induces chemotaxis of severe asthmatic BSM cells only

For all *in vitro* experiments, we used BE cells and BSM cells derived from 20 non-asthmatic subjects and 13 severe asthmatic patients (Table 1). We did not observe any specific orientation of the migration of the non-asthmatic BSM cells (Figure 1D), whereas the severe asthmatic BSM cells migrated significantly toward RV-infected BE supernatant (Figure 1E). The forward migration index was significantly higher in the severe asthmatic BSM cells than in the non-asthmatic BSM cells (0.27 ± 0.10 vs 0.03 ± 0.01 , respectively, $p=0.008$) toward RV-infected BE supernatant (Figure 1F). By contrast, both PDGF, an established activator of BSM cell migration, and non-infected BE supernatant induced similar migration of the non-asthmatic and severe asthmatic BSM cells (see Figure S3A-C and Figure S4 in the Online Repository).

RV-infected BE produces high levels of CXCL10

Using an unbiased transcriptomic approach, we identified 10 cytokines with a log₂-fold increase higher than 5 (see Table S3 in the Online Repository) in relation to the important inflammatory response of BE following RV infection. Notably, CXCL10 and CXCL9 mRNA expression had the highest significant fold change in response to RV infection (log₂ fold change: 13 and 11.2 respectively) (Figure 2A). Using ELISAs, we demonstrated that RV infection of BE significantly increased both the CXCL10 and CXCL9 protein concentrations in the supernatant (Figure 2B). To correlate these findings to the *in vivo* situation, we analyzed the CXCL10 and CXCL9 protein concentration in the serum of 51 severe asthmatic patients either in a stable state or during an acute exacerbation occurring within the RV circulation peak (patient's characteristics during stable state are available in Table S2 in the Online Repository). The concentration of CXCL9 was unchanged

whereas that of CXCL10 was significantly increased in the serum of these 51 severe asthmatic patients during exacerbation compared to the stable state (Figure 2C). Furthermore, we also compared the level of CXCL10 both in stable state or during an acute exacerbation according to the asthma phenotype or the level of various inflammatory markers, assessed at stable state. We did not find any significant difference in the level of serum CXCL10 between allergic and non-allergic patients, Type 2 and non-Type 2 patients, eosinophilic and non-eosinophilic patients (Data not shown). Similarly, there was also no difference in the level of serum CXCL10 between patients with total IgE higher and lower than 75 or 150 kUI/L, blood eosinophils higher and lower than 150 cell/ μ l, FeNO higher and lower than 20 or 50 ppb (Data not shown). These negative results suggested that the increase in serum CXCL10 during an acute asthma exacerbation occurring within the RV circulation peak was independent of the severe asthma phenotypes.

Moreover, using ELISA and BE cells derived from severe asthmatic patients, we demonstrated that RV infection significantly increased the CXCL10 protein concentrations in the supernatant to a similar level of that induced by the RV in BE cells derived from non-asthmatic subjects (see Figure S5A in the Online Repository).

As BSM cells are also able to produce CXCL10²⁵, we assessed the level of CXCL10 in the supernatant of BE co-cultured with either severe asthmatic or non-asthmatic BSM cells (see Figure S5B in the Online Repository). Interestingly, whereas low level of CXCL10 is detectable in both co-culture conditions suggesting that BSM cells are secreting basal level of CXCL10, RV infection of the BE induced an important secretion of CXCL10. However, the levels of CXCL10 were similar to those measured without BSM cells. Altogether, these results suggest that RV infection is the important trigger of CXCL10 production independently of either the origin of the BE or the co-culture with BSM cells.

Migration of severe asthmatic BSM cells toward RV-infected BE is CXCR3-dependent

Whereas recombinant CXCL10 was unable to induce any oriented migration of non-asthmatic BSM cells, CXCL10 significantly induced migration of severe asthmatic BSM cells (see Figure S3D-F in the Online Repository). Moreover, a specific blocking antibody against the CXCL10-specific receptor (*e.g.*, CXCR3) abrogated the migration of severe asthmatic BSM cells toward both the CXCL10 gradient (see Figure S3F in the Online Repository) and RV-infected BE supernatant (Fig 2D-F). Not surprisingly, the CXCR3 blocking antibody did not alter the migration of non-asthmatic BSM cells (see Figure S3G-I in the Online Repository).

Expression of CXCR3-B is decreased in severe asthmatic BSM cells

We did not find any difference in the total BSM protein expression of pan-CXCR3 between non-asthmatic subjects and severe asthmatic patients using western blots (Figure 3A). In contrast, the total expression of the specific isoform CXCR3-B was significantly decreased in BSM cells from severe asthmatic patients compared to those from non-asthmatic subjects (Figure 3B). Similarly, the cell surface expression of pan-CXCR3 was not different between the populations assessed by flow cytometry (Figure 3C), whereas that of the specific isoform CXCR3-B was significantly decreased in severe asthmatic BSM cells compared to non-asthmatic BSM cells (Figure 3D). To assess whether such a difference in CXCR3-B expression was specific to BSM, we analyzed the membrane expression of both pan-CXCR3 and CXCR3-B in CD3⁺ T cells from the severe asthmatic patients and non-asthmatic subjects. Interestingly, we did not find any difference in either pan-CXCR3 or CXCR3-B membrane expression in CD3⁺ T cells from the severe asthmatic patients or non-asthmatic subjects (see Figure S6 in the Online Repository).

Using immunocytochemistry, we morphologically confirmed the decreased expression of the CXCR3-B isoform in severe asthmatic BSM cells, whereas pan-CXCR3 staining was similar in severe asthmatic and non-asthmatic BSM cells (Figure 3E). Finally, using total proteins extracted from BSM tissue dissected *ex vivo*, we also observed significantly decreased expression of the CXCR3-B isoform in severe asthmatic BSM compared to non-asthmatic BSM (Figure 3F). Using RT-PCR, we did not demonstrate any difference in mRNA levels of both CXCR3-A and CXCR3-B between severe asthmatic and non-asthmatic BSM (data not shown).

Signaling pathways of CXCR3-A and CXCR3-B are altered in asthmatic BSM cells

We then investigated the downstream pathways associated with CXCR3-A or CXCR3-B activation by CXCL10 in both severe asthmatic and non-asthmatic BSM cells. We first analyzed the CXCL10-induced calcium response, which is known to be CXCR3-A-dependent in other cell types such as pneumocytes²⁶. Both the percentage of responding cells and the maximal calcium response to CXCL10 were increased in the severe asthmatic BSM cells compared to the non-asthmatic BSM cells (Figure 4A-C). We then examined PKA and PKC, which are known to be related to CXCR3-B activation in endothelial or vascular smooth muscle cells²⁷⁻³⁰. We clearly demonstrated that CXCL10 induced lower PKA expression (Fig 4D) and lower PKC (Figure 4E) activation in the severe asthmatic BSM cells than in the non-asthmatic BSM cells.

DISCUSSION

Taken together, these results demonstrate that BSM cells from severe asthmatic patients migrate toward the RV-infected epithelium. Such a specific migratory response is related to an increase in CXCL10 production by RV-infected epithelium both *in vitro* and *in vivo*, as well as to a decrease in CXCR3-B isoform protein expression and activation in severe asthmatic BSM cells.

In this study, we focused on the mechanisms of BSM cell migration related to BSM remodeling in severe asthma. Indeed, smooth muscle remodeling is a key event in diseases such as vascular diseases (*e.g.*, atherosclerosis or pulmonary hypertension)^{31, 32}, where smooth muscle cell migration has been extensively studied and shown to be a major mechanism^{33, 34}. In asthma, by contrast, very few studies have examined the role of cell migration in BSM remodeling^{35, 36}. For instance, Saunders *et al.* showed, in a proof of concept trial, that a DP2 receptor antagonist (*e.g.*, fevipiprant) reduced both BSM mass *in vivo* and BSM cell migration *in vitro* in asthmatic patients³⁷, identifying migration as a key mechanism of BSM remodeling. Since the rate of exacerbations is an important feature of severe asthma, we assessed the effect of RV infection of BE on severe asthmatic BSM cell migration toward infected epithelium. Here, we clearly demonstrate that RV-infected BE induced the migration of BSM cells from severe asthmatic patients but not that of BSM cells from non-asthmatic subjects. Shariff *et al.* previously demonstrated that RV-infected basal BE cells induced the migration of non-asthmatic BSM cells²⁰. However, in this study they used a MOI of 1 compare to 0.1 in the present study to better mimic an *in vivo* infection and we used fully differentiated BE cultured at the air-liquid interface, which is more relevant to asthma pathophysiology³⁸. Moreover, we used a more relevant cell migration assay that assessed the competitive migration of BSM cells between non-infected and RV-infected BE culture medium.

Finally, such a setup, compared to the classic Boyden chamber assay, generates a stable gradient and recording of random migration, which can be distinguished from primed and directed migration³⁹. Moreover, when assessing specific migration of BE supernatant only, without competition with infected BE supernatant (Figure S4 in the Online Repository), we were able to confirm Sharif et al findings that BE supernatant induced non-asthmatic BSM cell migration. Since we did not challenge BE with RV at various MOI (*e.g.* from 0.1 to 1), it is impossible to demonstrate a MOI-dose-dependent behavior, which should be further assessed in the migration of both severe asthmatic and non-asthmatic BSM cells.

In a different study performed by Kuo *et al*, direct RV infection of BSM cells from asthmatic patients induced an increased migration compare to non-asthmatic BSM cells, through an increase of extracellular matrix deposition (*i.e.* Fibronectin and perlican)³⁶. Whereas in this study the effect of RV infection was assessed directly on BSM cells and not through epithelial infection as in the present study, the increase of asthmatic BSM cell migration compare to non-asthmatic BSM cell migration was also demonstrated in this context³⁶.

We demonstrated that CXCL10 induced the migration of BSM cells from severe asthmatic patients only. However, CXCL10 has been shown to be involved in both bronchial inflammation and hyperresponsiveness in a mouse model of asthma⁴⁰. CXCL10 is a major chemokine produced by BE in response to several pathogens, such as RV, respiratory syncytial viruses^{41, 42} and house dust mite allergens⁴³. We confirmed the production of CXCL10 in the present model of fully differentiated BE in response to RV infection. We used a low MOI (*e.g.*, 0.1) for both physiological and technical reasons. On the one hand, such a low MOI emulated an *in vivo* infection of BE⁴⁴, and on the other hand, an MOI of 0.1 did not allow any leakage of RV particles in the co-culture medium used to stimulate BSM cell migration. Indeed, we did not find any RV RNA particles in

the co-culture medium using digital PCR (data not shown). Previous studies have already shown an increase in CXCL10 expression in bronchoalveolar lavage fluid, lung tissue and serum from asthmatic patients compared to non-asthmatic subjects^{43, 45}. However, we report here, for the first time, that CXCL10 was significantly increased in the serum of severe asthmatic patients during an exacerbation compared to the same patients in a stable state. Moreover, when comparing the level of CXCL10 according to asthma phenotypes¹ and various inflammatory markers (Total IgE, blood eosinophils, FeNO levels), we did not find any differences at both basal state and during an exacerbation. These important negative results highlight that the increased CXCL10 during an exacerbation is not restricted to one phenotype of severe asthma.

CXCL10 specifically binds to the chemokine receptor CXCR3⁴⁶. This receptor, is expressed by various cell types, such as lymphocytes, mast cells, eosinophils or neutrophils⁴⁷⁻⁵⁰ and has also been identified in BSM cells⁵¹. We confirmed this result using both western blotting and flow cytometry. Moreover, we demonstrate, for the first time, the role of CXCR3 in asthmatic BSM cell migration toward RV-infected BE using a blocking antibody against CXCR3. Splicing of CXCR3 mRNA leads to two isoforms, CXCR3-A and CXCR3-B⁴⁶. In the present study, we show that the protein expression of the CXCR3-B isoform was decreased in BSM cells from severe asthmatic patients compared to those from non-asthmatic subjects, whereas that in T cells remained unchanged. To the best of our knowledge, this specific decreased expression of CXCR3-B in severe asthmatic BSM cells has never been reported. Interestingly, CXCR3-A is known to act as an activator of cell migration, whereas CXCR3-B is an inhibitor of this migration^{46, 52}. We demonstrate that CXCL10 predominantly activates the CXCR3-A pathway in severe asthmatic BSM cells, unlike in non-asthmatic BSM cells. Indeed, the intracellular calcium response, which has been related to CXCR3-A activation in other cell types such as prostate cancer cells or vascular

pericytes^{53, 54}, was increased in BSM cells from severe asthmatic patients. In contrast, CXCL10 predominantly activates CXCR3-B in non-asthmatic BSM cells compared to severe asthmatic BSM cells, resulting in PKC and PKA activation, as previously identified for example in endothelial cells^{27, 30}.

Finally, we note some limitations of this study. First, since we only collected bronchial biopsies, we were not able to analyze BSM remodeling of the entire bronchial wall, as previously performed by Pascoe *et al*⁵⁵. However, this complete histological analysis requires lung specimens, which were not available for our severe asthmatic patients. In the present study, we analyzed the basal membrane to BSM distance at regular intervals of 50 μ m using validated method²². As previously described²², we identified a clear and significant difference between severe asthmatic patients and non-asthmatic subjects, with a low heterogeneity between patients. By contrast, Pascoe *et al*, identified very high heterogeneity in bronchial remodeling⁵⁵. Thus, basal membrane to BSM distance measurements would require confirmation using whole airway histology in the future. Second, we decided to use BE cells from non-asthmatic subjects only. Indeed, we focused our attention on asthmatic BSM remodeling. In this regard, we previously demonstrated that the increased proliferative response of asthmatic BSM cells induced by house dust mite-stimulated BE cells was not related to the asthmatic origin of BE cells²⁴. Third, when examining the CXCL10 concentration in the serum of exacerbating patients, we did not concomitantly assess the presence of RV in the sputum. However, we focused on collecting serum samples of severe asthmatic patients undergoing exacerbation during the RV circulation epidemic period (*i.e.*, from October to February in Europe)⁵⁶ in the absence of bacterial infection and serum samples of stable patients outside the RV circulation period (*i.e.*, from April to August). Further studies are needed to confirm

that documented RV exacerbations significantly increase CXCL10 concentrations in serum from severe asthmatic patients. Moreover, such an increase of CXCL10 level may not be restricted to BSM cell migration but could participate to the recruitment of various inflammatory cells during asthma exacerbations ⁴⁰. Fourth, as we specifically assessed the migration of BSM cells derived from severe asthmatic patients compare to non-asthmatic patients, the levels of asthma medication such as corticosteroids and long acting bronchodilators were different between both populations and may have modulated CXCR3 expression, as previously described with acute treatment in mice ⁵⁷. However, the BSM cells used in this study were first cultured from patients' biopsies during at least two months. This long duration would allow, in theory, the inactivation of all pharmacological compounds. Fifth, we did not analyze the CXCR3-A isoform by western blots or flow cytometry, since there is no available dedicated antibody because the unique difference between CXCR3-A and CXCR3-B isoforms is the presence of 52 additional amino acids in the N-terminus of the CXCR3-B isoform ⁴⁶. However, to avoid this technical issue, we also assessed downstream signaling pathways of both isoforms in response to CXCL10 stimulation. Finally, we only focused our study on severe asthmatic patients and did not analyze BSM cells from mild asthmatic patients. Whereas BSM remodeling is an important feature of severe asthma, some mild asthmatic patients also exhibit a significant BSM remodeling⁵. The role of the CXCL10/CXCR3 axis has never been assessed in these mild asthmatic patients. Thus, further analyses will be needed to assess the specificity of this migration mechanism in the severe asthmatic population.

To conclude, the present findings describe a potential novel mechanism of BSM remodeling in severe asthmatic patients. This study demonstrates that severe asthmatic BSM cells are intrinsically different from non-asthmatic BSM cells with an imbalance in CXCR3-A/CXCR3-B.

449 Indeed, CXCR3-A signaling was increased and both CXCR3-B expression and signaling were
450 decreased in asthmatic BSM cells. Thus, we suggest that the CXCL10/CXCR3-A axis could be a
451 new specific therapeutic target for BSM remodeling in severe asthma.

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Journal Pre-proof

ACKNOWLEDGEMENTS

We thank the staff of the pathology and surgery departments at the University Hospital of Bordeaux, as well as Isabelle Goasdoue, Virginie Niel and Marine Servat from the Clinical Investigation Center for technical assistance and all the team of the Bordeaux Imaging Center (BIC, Bordeaux University). Regarding project funding, we strongly acknowledge the “Agence Nationale pour la Recherche” (ANR, ROSAE project CE14-0015-01), the “Fondation de l’Université de Bordeaux” from the FGLMR/AVAD grant and an unrestricted grant from AstraZeneca.

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Table 1: Clinical and functional characteristics of subjects

Characteristics	Non-asthmatic subjects	Asthmatic patients	<i>P</i> value
No. of patients	20	13	
M/F sex ratio	14/6	5/8	0.1
Age, yr	61.81 ± 2.10	53.06 ± 4.53	0.3
Range, yr	42-73	31-72	
Body mass index, kg/m ²	26.75 ± 0.97	26.32 ± 1.38	0.9
Treatments			
LABA, no. of patients	0	13	< 0.001
ICS, no. of patients	0	13	< 0.001
OCS, no. of patients	0	4	0.02
FEV1			
Liters	2.73 ± 0.14	1.88 ± 0.17	0.002
Percentage of predicted value	93.05 ± 4.21	75.88 ± 6.35	0.04
FVC			
Liters	3.76 ± 0.20	2.73 ± 0.18	0.02
FEV1/FVC Ratio	0.73 ± 0.02	0.68 ± 0.03	0.4

Data are presented as mean ± SEM for continuous normal variables. Comparisons of continuous variables were made with either Mann-Whitney or t-tests. Categorical variables were analysed with Fisher's exact tests. BMI= body mass index; ICS= inhaled corticosteroids; LABA= long-

acting beta2-agonist; OCS: continuous oral corticosteroids; FEV₁= forced expiratory volume in one second, FVC= forced volume capacity.

FIGURES LEGENDS

Figure 1: (A-B) Representative microscopic images of immunohistochemistry stained with anti-alpha-smooth muscle actin antibody in bronchial sections obtained from a non-asthmatic subject **(A)** and severe asthmatic patient **(B)**. Scale bar is 100µm. **(C)** Measure of the distance between epithelial basal membrane and smooth muscle in non-asthmatic subjects (n=11) and severe asthmatic patients (n=24). **(D-E)** Representative BSM cell migration tracks over 24h obtained from a non-asthmatic subject **(D)** and severe asthmatic patient **(E)** in response to RV-infected epithelium. **(F)** Forward migration index (FMI) of BSM cells obtained from non-asthmatic subjects (n=5) and severe asthmatic patients (n=5) in response to RV-infected epithelium. Data are presented as individual dots with their median (horizontal line). Comparisons were performed using Mann-Whitney tests. *, P < 0.05.

Figure 2: (A) Volcano plots of chemokine and cytokine genes differentially expressed in epithelial cells in response to rhinovirus infection using NanoString technology. Results presented are obtained from 4 different epithelial cells. Y axis line at y= 1.3 represent a p=0.05 of the fold change

in RV-infected epitheliums. X axis lines at $x = 2.32$ and $x = -2.32$ represent a fold change of 5 times in RV-infected epitheliums. Red dots represent CXCL9 and CXCL10. **(B)** CXCL9 and CXCL10 concentration measured by ELISA in basal supernatant of epithelium with and without RV infection ($n=5$ and 6 respectively). **(C)** CXCL10 concentration measured by ELISA in serum of severe asthmatic patients ($n=51$) in stable state (S) and during an exacerbation (Exa). **(D-F)** Effect of CXCL10 gradient on BSM cell migration. **(D-E)** Representative BSM cell migration tracks over 24h obtained from a non-asthmatic subject **(D)** and a severe asthmatic patient **(E)** in response to CXCL10 gradient. **(F)** Forward migration index (FMI) of BSM cells obtained from severe asthmatic patients ($n=4$) in response to CXCL10 gradient after incubation with either irrelevant or CXCR3 blocking antibody. Data are presented as individual dots with their median (horizontal line). Comparisons were performed using Mann-Whitney or Wilcoxon matched paired tests. *, $P < 0.05$.

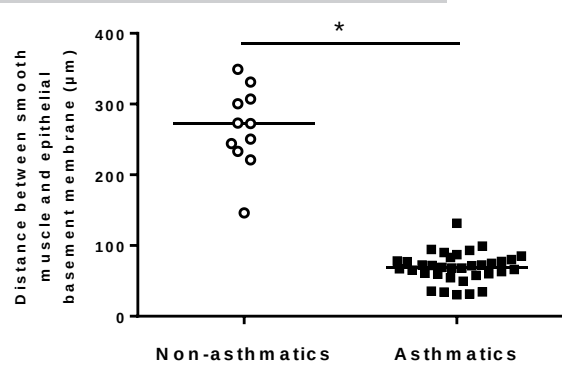
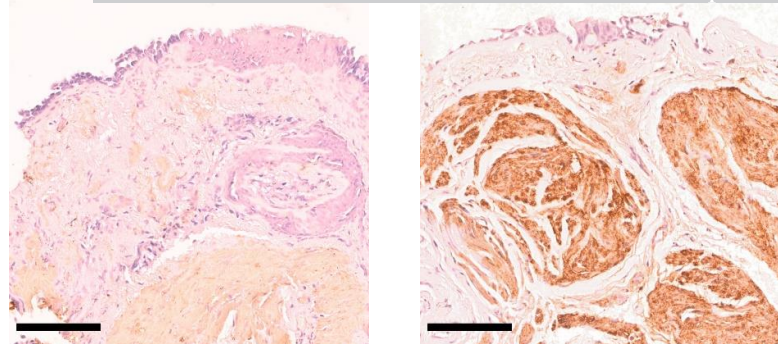
Figure 3: (A-B) Pan-CXCR3 **(A)** and CXCR3-B **(B)** protein expression assessed by western blot in BSM cells obtained from non-asthmatic subjects ($n=4$) and severe asthmatic patients ($n=4$). Total protein represents a portion of the “stain-free” images used for normalization. **(C-D)** Pan-CXCR3 **(C)** and CXCR3-B **(D)** membrane expression assessed by flow cytometry in BSM cells obtained from non-asthmatic subjects ($n=5$) and severe asthmatic patients ($n=5$). **(E)** Representative images of immunocytochemistry with CXCR3-pan and CXCR3-B antibodies on BSM cells from non-asthmatic subjects and severe asthmatic patient. **(F)** CXCR3-B expression assessed by western blot in BSM tissue obtained from biopsies of non-asthmatic subjects ($n=5$) and severe asthmatic patients ($n=5$). Data are presented as individual dots with their median (horizontal line). Comparisons were performed using Mann-Whitney tests. *, $P < 0.05$.

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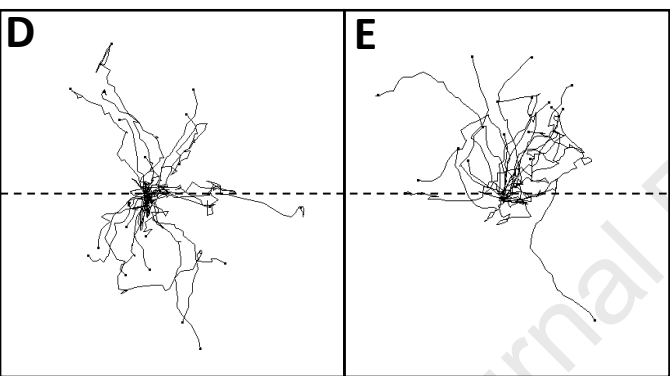
667 **Figure 4: (A)** Representative calcium response of either a single BSM cell from non-asthmatic
668 subject (black hashed line) or severe asthmatic patient (black line). Variations of relative cytosolic
669 Ca^{2+} (F/F0) concentration were monitored by fluorescence video microscopy in fluo4-loaded
670 CXCL10 (100 ng/ml) was added at 16 s (as indicated by the arrow). **(B)** Percentages of responding
671 cells and **(C)** amplitude of the calcium rises in response to CXCL10 (White dots, n=x cells from 5
672 different patients; Black dots, n=y cells from 4 different patients). **(D)** Fold change expression of
673 PKA catalytic subunit after 1h stimulation with 100ng/mL CXCL10 in BSM cells from non-
674 asthmatic subjects (White dots, n=5) and severe asthmatic patients (Black dots, n=5). **(E)** Fold
675 change expression of phosphorylated PKC (Thr 514) after 1h stimulation with 100 ng/mL
676 CXCL10 in BSM cells from non-asthmatic subjects (White dots, n=6) and severe asthmatic
677 patients (Black dots, n=7). Data are presented as individual dots with their median (horizontal line)
678 or as bars. Comparisons were performed using Mann-Whitney or χ^2 tests. *, $P < 0.05$.

679

A



RV-infected epithelium supernatant



Epithelium supernatant

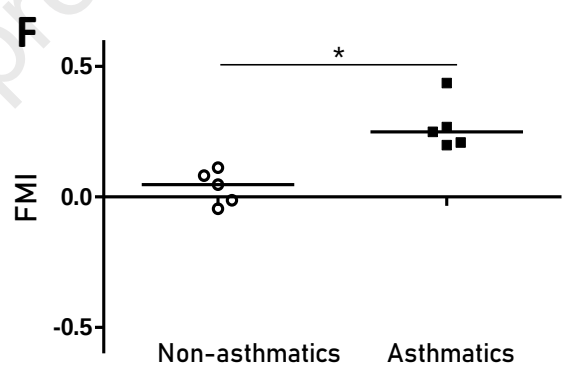


Figure 1

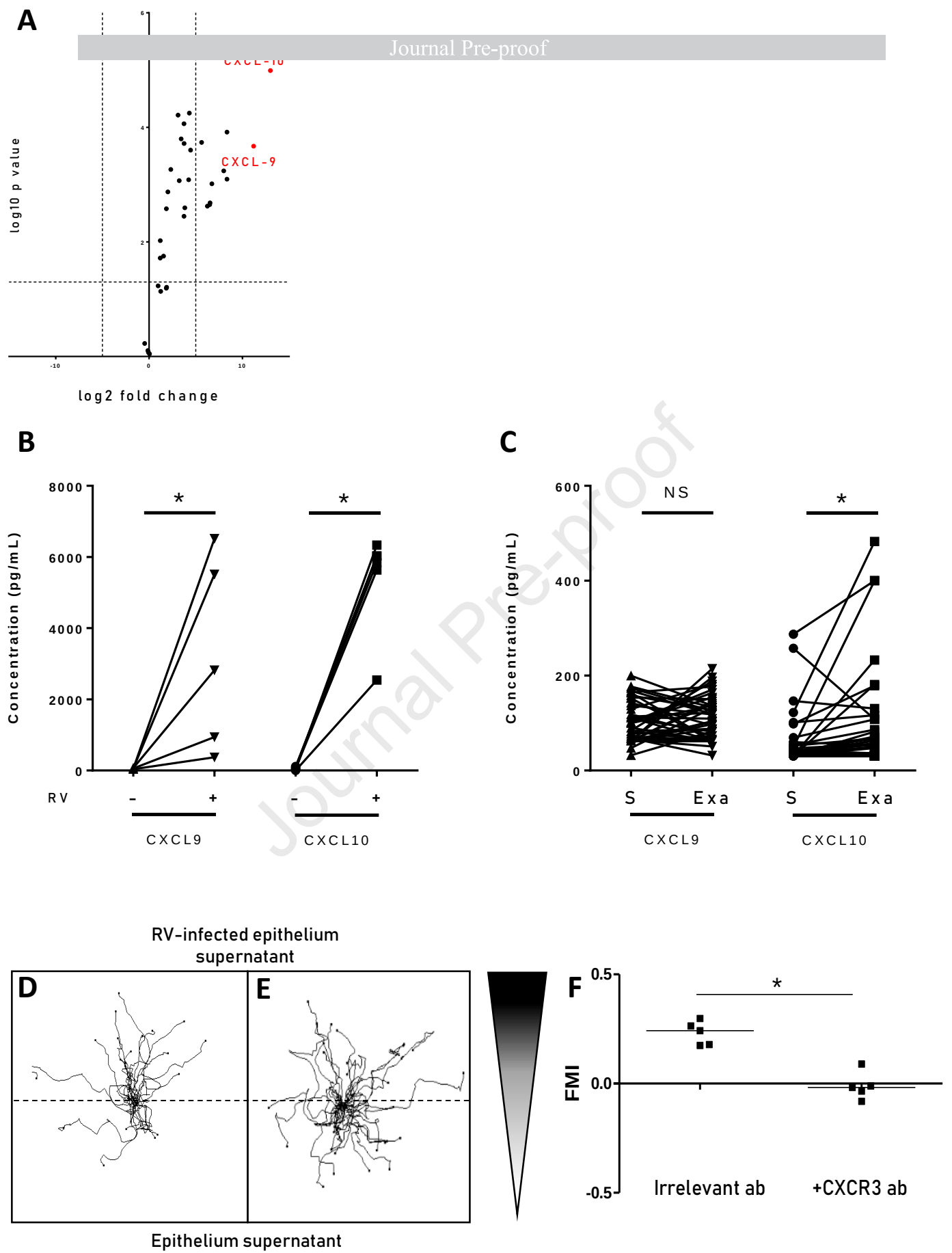


Figure 2

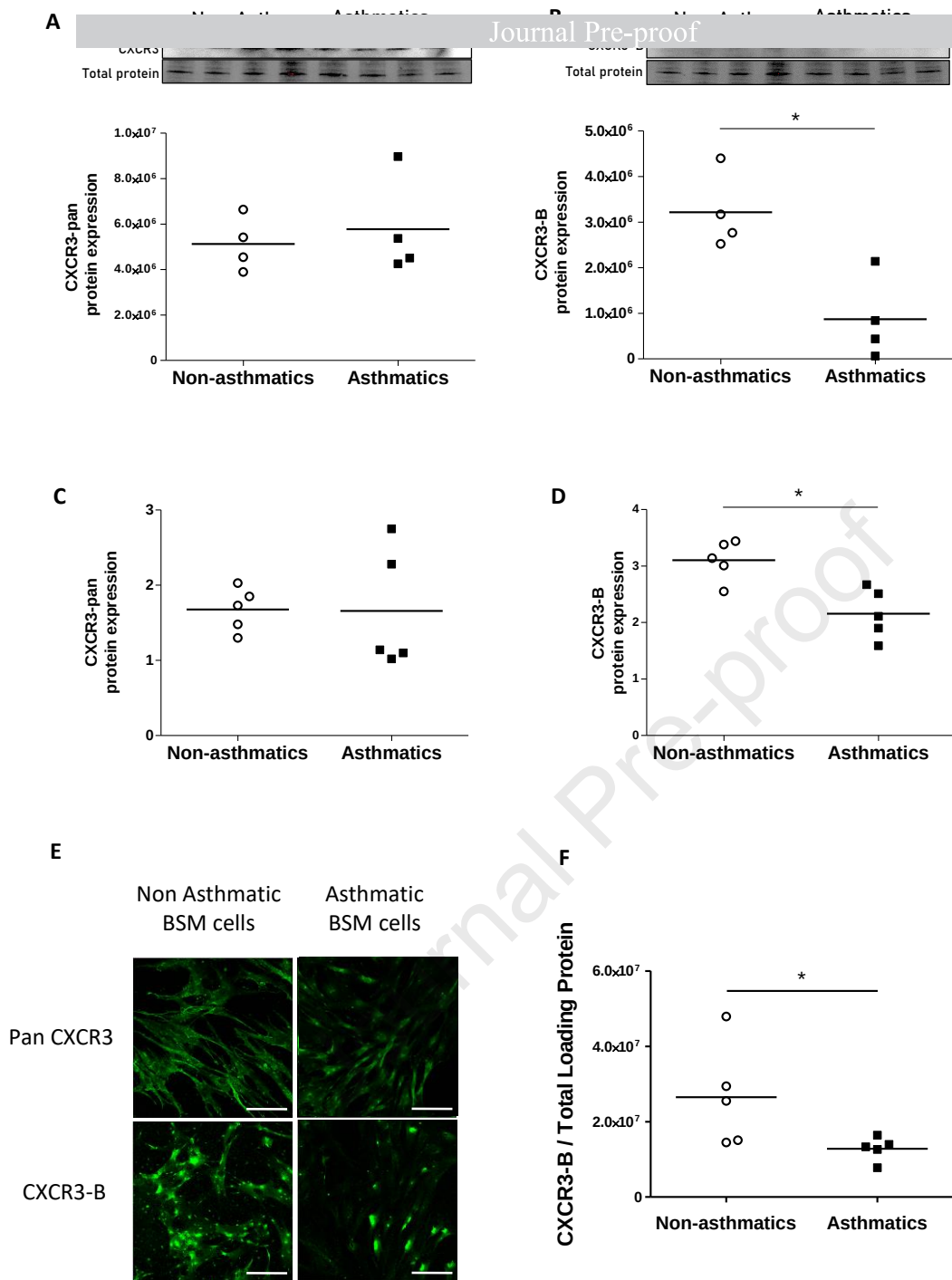


Figure 3

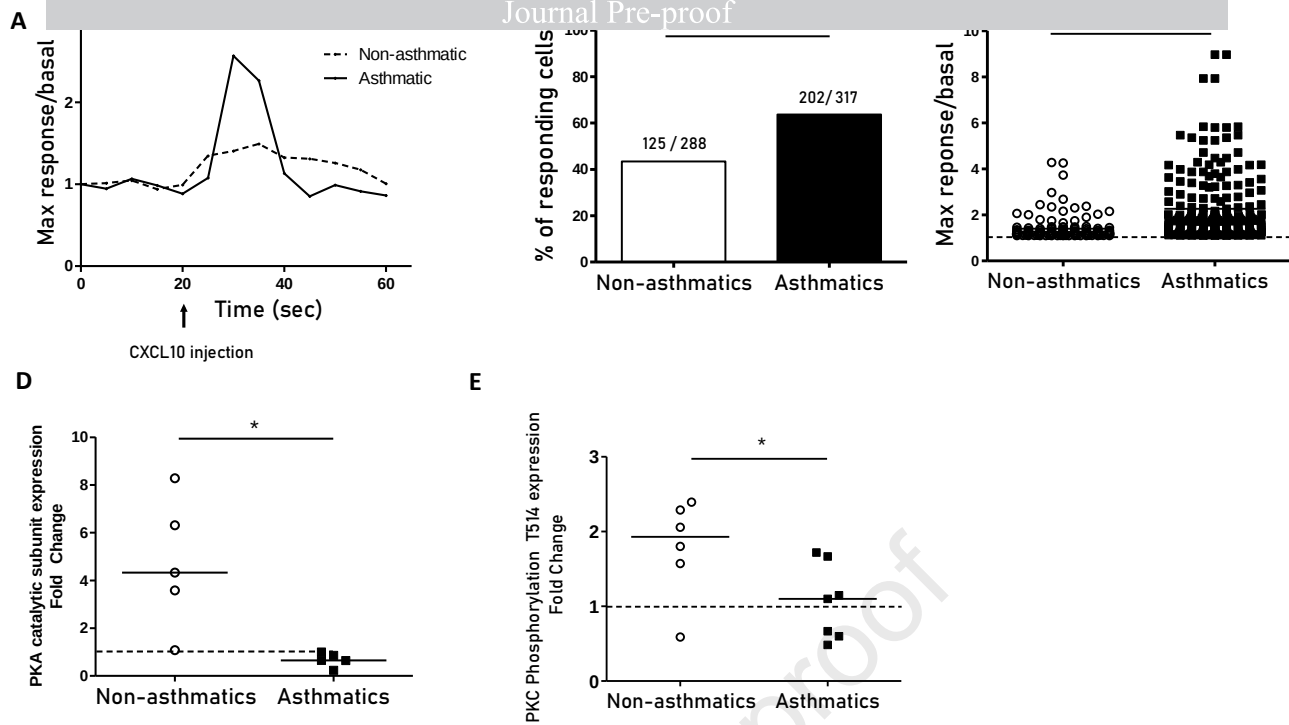


Figure 4