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IL-33 promotes the migration and proliferation of circulating fibrocytes from patients with allergen-exacerbated asthma

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ABSTRACT

The release of IL-33 increases in the bronchial mucosa of asthmatic patients in relation to disease severity and several studies have demonstrated that IL-33 may enhance airway inflammation in asthma. This study tested the hypothesis that IL-33 may also contribute to the development of irreversible structural changes in asthma by favoring the airway recruitment and profibrotic function of circulating fibrocytes during episodes of allergen-induced asthma exacerbation. The circulating fibrocytes from patients with allergen-exacerbated asthma (PwAA) showed increased expression of the specific IL-33 receptor component ST2L in comparison with the cells from non-asthmatic individuals (NAI). Recombinant IL-33 induced the migration of circulating fibrocytes from PwAA at clinically relevant concentrations and stimulated their proliferation in a concentration-dependent manner between 0.1 and 10 ng/ml, without affecting the constitutive release of type I collagen. The recombinant protein did not induce similar responses in circulating fibrocytes from NAI. This study uncovers an important mechanism through which fibrocytes may accumulate in the airways of allergic asthmatics when their disease is not adequately controlled by current treatment and provides novel information on the function of IL-33 in asthma.

1. Introduction

The fibrocytes were originally identified in cultures of peripheral blood mononuclear cells (PBMCs) and in the wounds as $CD45^+$ $CD45RO^+CD34^+CD11b^+CD13^+HLA\text{-}DR^+$ cells that constitutively produce fibronectin and collagens and express the myofibroblast marker α-smooth muscle actin upon stimulation with TGF-β_1 [1–3]. There is increasing evidence that these cells are circulating mesenchymal progenitors, serving as a renewable source of fibroblasts and myofibroblasts in the repair of extensive tissue injury and in fibrotic disorders associated with chronic inflammation [4]. A substantial proportion of bronchial myofibroblasts in asthma derives from circulating fibrocytes and fibrocyte infiltration of chronically-inflamed asthmatic airways likely contributes to the development of subepithelial fibrosis [5–9]. The mechanisms involved in this profibrotic function of fibrocytes should be investigated because subepithelial fibrosis is scarcely responsive to treatment and may cause irreversible airflow obstruction [10].

Factors released from the bronchial epithelium of asthmatic patients may promote the airway recruitment and proliferation of fibrocytes because these cells preferentially infiltrate the subepithelial zone [5,6,8]. One of the epithelial cell-derived factors produced in excessive amounts in asthma is IL-33 [11,12], a member of the IL-1 family exhibiting a dual function similar to that of other endogenous danger signals [13–17]. The IL-33 released from injured or biomechanically strained cells [16,18–20] works as a classical cytokine by binding to a heterodimeric receptor complex that consists of the trans-membrane specific component with restricted expression, ST2L, and the broadly expressed IL-1 receptor accessory protein (IL-1RAcP) [21,22].

As extensively reviewed elsewhere [11,12], the synthesis and release of IL-33 in asthmatic airways increase in relation to disease severity and numerous studies have suggested that released IL-33 may enhance airway inflammation in asthma. Some recent observations [23,24] have however uncovered potential profibrotic properties of IL-33 that may be relevant to asthma. This study

Abbreviations: Ab, antibody; COL1, type I collagen; IL-1RAcP, IL-1 receptor accessory protein; m, monoclonal; NAI, non-asthmatic individuals; PBMCs, peripheral blood mononuclear cells; PwAA, patients with allergen-exacerbated asthma; r, recombinant.

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provides the first evidence that IL-33 may contribute to the progression of structural changes in asthmatic airways by favoring the recruitment and proliferation of circulating collagen-producing fibrocytes during each episode of disease exacerbation.

2. Materials and methods

2.1. Selection of fibrocyte donors and blood sampling

Fibrocyte donors were 9 patients with allergen-exacerbated asthma (PwAA) and 14 non-asthmatic individuals (NAI) (Supplemental Table 1) who gave informed consent following protocol approval by the relevant review board. The selection of PwAA as fibrocyte donors was based on the results of previous studies [5,9] that demonstrated the presence of high numbers of fibrocytes both in the peripheral blood and in the airways of all asthmatics with an induced or spontaneous worsening of the disease provoked by allergen inhalation. All patients had a history of persistent asthma with previously documented reversible airflow obstruction and airway responsiveness to inhaled methacholine according to international guidelines [25]. The atopic status had been previously assessed by skin prick testing and all patients had already suffered from similar episodes of asthma worsening during natural exposure to one or more of the allergens to which they were sensitized. The NAI had no history of allergic or respiratory diseases and their lung function was normal, as assessed by spirometry. Their atopic status was however evaluated by skin prick testing on a separate day, using a battery of common allergens. Spirometry was performed in all subjects according to standard procedures [26] and the asthmatic patients were asked to withhold bronchodilators for 24 h before testing, if possible. Venous blood samples were collected on the same visit, before spirometry, and processed to isolate circulating fibrocytes as described below.

2.2. Isolation and flow cytometry analysis of circulating fibrocytes

$CD34^+$ cells were separated from PBMCs by using the Dynal CD34 Progenitor Cell Selection System (Invitrogen). After detachment of the immunomagnetic beads, the recovered $CD34^+$ cells were repeatedly washed and then stained with Pacific Blue-CD45RO monoclonal (m) antibody (Ab) (BioLegend), PE-CD11b mAb (Abcam) and PerCP-eFluor 710-CD13 mAb (eBioscience). The $CD34^+$ cells that co-expressed CD45RO, CD11b, and CD13 were sorted on a BD FACSAria II (BD Biosciences) and the gates were set using isotype-matched irrelevant Abs as negative controls (Supplemental Fig. 1). The purity of the sorted cells was ascertained by double staining for CD34 and intracellular type I collagen (COL1) (Supplemental Fig. 1). For CD34 staining, an APC-CD34 mAb (StemCell Technologies) was used. For intracellular staining, cells were fixed/permeabilized with the Cytofix/Cytoperm kit (BD Biosciences) and either sequentially incubated with rat anti-COL1 (α1 chain) (Chemicon International) and a FITC-labeled goat anti-rat secondary Ab (Invitrogen) or treated with a mouse anti-COL1 (LifeSpan BioSciences) after mAb labeling with EasyLink FITC Conjugation Kit (Abcam). Samples of the sorted cell population were then evaluated for the surface expression of ST2L and IL-1RAcP by staining with a FITC-ST2 mAb (MBL International), the anti-ST2 mAb conjugated to APC (LNK031APC, AbD Serotec) or an APC-IL-1RAcP mAb (R&D Systems). The cells stained with these Abs or the appropriate controls was analysed using a FACSCalibur flow cytometry system and the CellQuest software (BD Biosciences). Before further testing, circulating fibrocytes were re-suspended in low-serum medium (RPMI 1640 medium containing 1% heat-inactivated FCS, 10 mM HEPES, 1% non-essential amino acids, 1% sodium pyruvate, 2mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin) and incubated overnight in Corning flasks coated with ultra-low attachment surface at 37 °C in a humidified atmosphere (5% CO_2 in air).

2.3. Expression of the mRNAs for ST2L and IL-1RAcP

Gene expression analysis was performed by quantitative RT-PCR. Specific primer pairs for the target genes and the reference gene *gapdh* were retrieved from relevant publications [23,27] and quantitative RT-PCR was conducted using a SYBR green-based cells-to-C_T kit (Applied Biosystems) as previously described [9]. The levels of expression of the mRNAs for ST2L and IL-1RAcP were normalized to the level of expression of the mRNA for GAPDH.

2.4. Chemotaxis assay

The chemotactic activities of recombinant (r)IL-33 (PeproTech) and the positive control, rIL-1β (R&D Systems) [3], were evaluated in a fluorescence-based, modified Boyden chamber assay with an uncoated 8-μm pore-size insert membrane (QCM Chemotaxis 96-Well Cell Migration Assay, Chemicon), according to the manufacturer's instructions. The fibrocytes isolated from the peripheral blood were placed into the upper compartment of the chemotaxis chamber (3×10^4 cells/100 μl assay medium/well) and allowed to migrate for 3 h in response to assay medium alone (RPMI 1640 medium supplemented with 10 mM HEPES, 1% nonessential amino acids, 1% sodium pyruvate, 2 mM L-glutamine and 0.1% BSA), medium containing 0.1–10 ng/ml of rIL-33, or medium containing 10 ng/ml of rIL-1β, at 37° C in a humidified incubator (5% CO_2 in air).

2.5. Proliferation assay

The mitogenic activities of rIL-33 and the positive control, rIL-1β [3], were estimated by direct fibrocyte enumeration. In some experiments, analysis of cell division was performed by recording the changes in fluorescence intensity of cells labeled with PKH-26, as previously reported [9]. Labeled or unlabeled fibrocytes were re-suspended in low-serum medium and seeded into the flat-bottom wells of standard 96-well plates (5×10^3 cells/well). Cells were incubated for 72 h in the presence or absence of rIL-33 (0.1–10 ng/ml) or rIL-1β (10 ng/ml), detached from the wells and enumerated by hemocytometry. PKH-26-labeled cells were permeabilized and incubated with the FITC-COL1 mAb or the isotype control before analysis by flow cytometry.

2.6. Release of proinflammatory and profibrotic factors

Fibrocytes were seeded into the flat-bottom wells of standard 96-well plates (10^4 cells/well) and incubated for 12 h in serum-free medium alone or in medium containing 0.1–10 ng/ml rIL-33. The conditioned media were assayed for determination of the contents of IL-6, IL-8/CXCL8, TGF-β_1 (ELISA kits from R&D Systems) and soluble COL1 (ELISA kit from MD Biosciences). The results of each assay were normalized to the cell number.

2.7. Inhibition of signaling through ST2L

The rIL-33-induced responses of circulating fibrocytes from PwAA were evaluated after pre-incubation of the medium containing a fixed dose of rIL-33 (10 ng/ml) with 0–200 ng/ml of rST2-Fc fusion protein (ALX-201–367) or a control Fc fusion protein (ALX-203–004) (both from Enzo Life Sciences) for 1 h at 37 °C.

2.8. Statistical analysis

This analysis was performed with GraphPad Prism for Windows (version 5). Significance was concluded when $p < 0.05$.

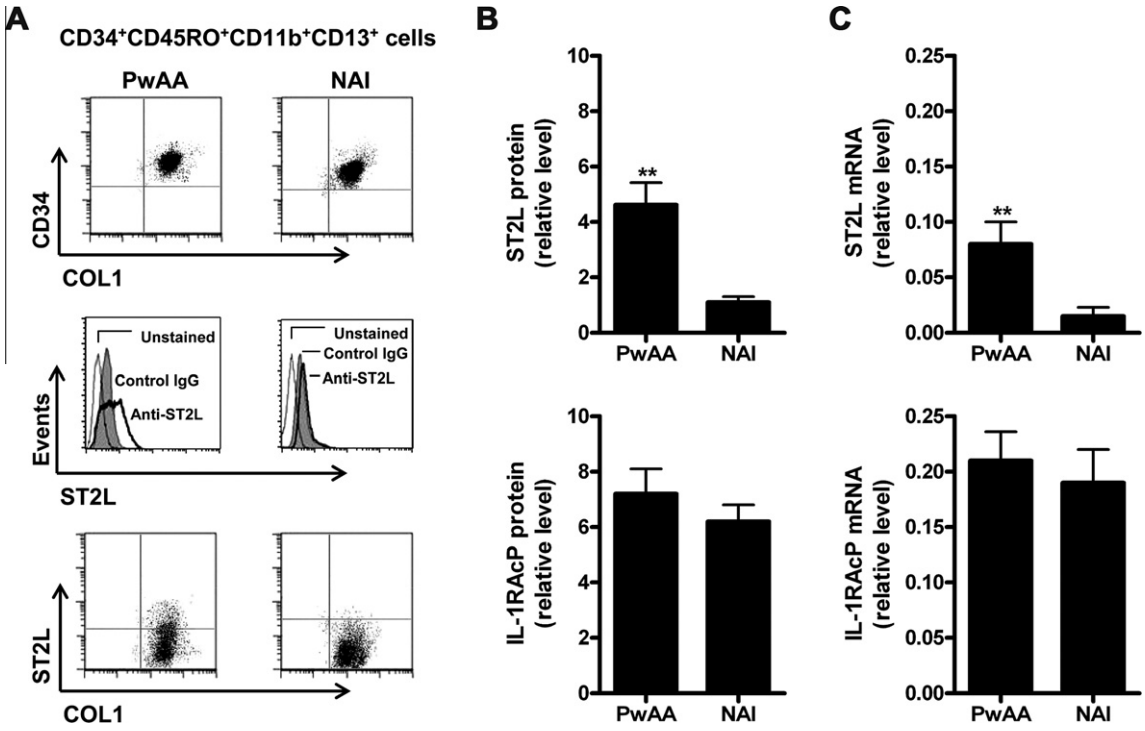

Fig. 1. Expression of the components of the IL-33 receptor in circulating fibrocytes from patients with allergen-exacerbated asthma (PwAA) and non-asthmatic individuals (NAI). (A) Representative flow cytometry analyses of circulating $CD34^+CD45RO^+CD11b^+CD13^+$ cells stained for CD34 and intracellular type I collagen (COL1), ST2L or ST2L and intracellular COL1. The horizontal and vertical lines in the dot plots mark fluorescence intensity greater than that observed with the isotype-matched controls for the specific monoclonal antibodies (APC-CD34, FITC-COL1 and APC-ST2). (B) Quantification of the relative levels of expression of surface ST2L and IL-1 receptor accessory protein (IL-1RAcP) proteins by flow cytometry. The median fluorescence intensity of cells stained with each specific monoclonal antibody was subtracted from that of cells stained with the corresponding isotype-matched control IgG and the obtained value was divided by the median fluorescence intensity of unstained cells. (C) Quantification of the relative levels of expression of the mRNA for ST2L and IL-1RAcP by quantitative RT-PCR. Data were normalized to the level of expression of the mRNA for GAPDH. Quantitative results are expressed as the means and SD. $^{**}p < 0.01$ compared with NAI by the unpaired Student's *t*-test; $n = 3–5$.

3. Results

3.1. Circulating fibrocytes from PwAA exhibited increased expression of ST2L

Fibrocytes were isolated from the PBMCs of PwAA and NAI on the basis of the known phenotypic characteristics of these cells [1–3,5,6,8] (Supplemental Fig. 1). The percentage of $COL1^+$ cells was $\geqslant$88.7%, without major differences between the two groups of individuals. In keeping with previous observations [9], the numbers of isolated fibrocytes per milliliter of blood were significantly higher in PwAA than in NAI ($p < 0.01$, Mann–Whitney *U* test), being the median (interquartile range) values 6.9×10^4/ml (5.3×10^4/ml) and 0.8×10^4/ml (1.6×10^4/ml), respectively.

Flow cytometry analysis of cells stained with specific mAbs against ST2L and IL-1RAcP revealed increased expression of ST2L in the circulating fibrocytes from PwAA (Fig. 1A and B) and no significant differences between groups for the expression of IL-1RAcP (Fig. 1B). By double staining for ST2L and intracellular COL1 it was possible to verify that the vast majority of the $ST2L^+$ cells from PwAA were indeed fibrocytes (Fig. 1A). The increased expression of ST2L in circulating fibrocytes from PwAA was confirmed at the mRNA level (Fig. 1C).

3.2. Stimulation with rIL-33 induced the migration and proliferation of circulating fibrocytes from PwAA

The ability of IL-33 to induce the migration and proliferation of circulating fibrocytes was tested by using the widely employed rIL-33 [16–20] that is known to contain a bioactive fragment of full length IL-33 (aa 112–270). Positive control was rIL-1β, the only member of the IL-1 family with demonstrated ability to induce the migration and proliferation of normal fibrocytes [3].

In a standard chemotaxis assay, the migration of circulating fibrocytes from PwAA significantly increased over the spontaneous migration in response to concentrations of rIL-33 as low as 0.1 ng/ml and further increased in response to higher concentrations of the recombinant protein (Fig. 2A). A significant migratory response was only observed when rIL-33 was added to the bottom compartment of the chemotaxis chamber (Fig. 2B), suggesting that the exogenous protein exerted a chemotactic effect and did not substantially increase the random motility of the cells (chemokinesis). The circulating fibrocytes from NAI did not show any migratory response on stimulation with 10 ng/ml rIL-33 (Fig. 2A) but they migrated in response to rIL-1β (Fig. 2C) as did the circulating fibrocytes from PwAA (Fig. 2C).

Recombinant IL-33 also induced the proliferation of fibrocytes from PwAA in a concentration-dependent manner (Fig. 3A). Its mitogenic effect was already evident at the concentration of 1 ng/ml and further increased at a ten-fold higher concentration (Fig. 3A). The circulating fibrocytes from NAI did not proliferate in response to stimulation with 10 ng/ml rIL-33 (Fig. 3A) although they showed a proliferative response to rIL-1β similar to that of circulating fibrocytes from PwAA (Fig. 3B). In some experiments, analysis of cell division was performed by recording the changes in fluorescence intensity of cells labeled with PKH-26, as previously reported [9]. This analysis confirmed that rIL-33 significantly stimulated the proliferation of circulating fibrocytes from PwAA (Fig. 3C). By staining the labeled cells for intracellular COL1, it was possible to ascertain that the proliferating cells were indeed fibrocytes (Fig. 3D).

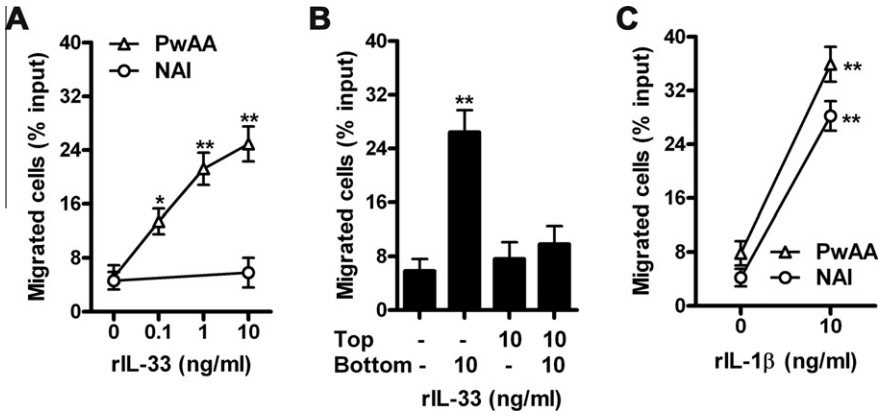

Fig. 2. Migration of circulating fibrocytes from patients with allergen-exacerbated asthma (PwAA) and non-asthmatic individuals (NAI). (A) Cell migration in response to rIL-33. (B) Effects of rIL-33 on the directed migration and random motility of circulating fibrocytes from PwAA. (C) Cell response to the control chemoattractant rIL-1β. Data are the mean number of migrated cells (expressed as percentage of the input cells) and SD. $^{**}p < 0.01$ and $^{*}p < 0.05$ compared with 0 ng/ml rIL-33 (A), 0 ng/ml rIL-1β C, or all the other experimental conditions (B) by analysis of variance followed by the Dunnett's or the Tukey's post hoc tests (A) and (B), and by the paired Student's *t*-test (C); $n = 3–5$.

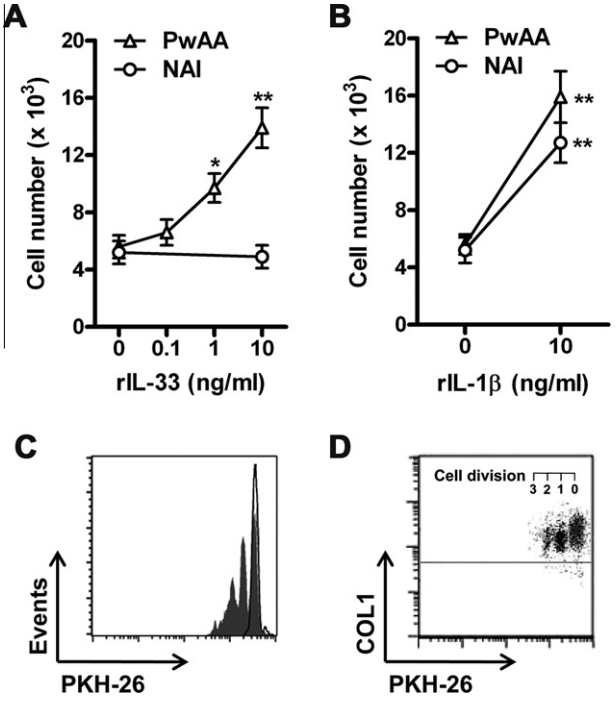

Fig. 3. Proliferation of circulating fibrocytes from patients with allergen-exacerbated asthma (PwAA) and non-asthmatic individuals (NAI). (A) Proliferative response to rIL-33. (B) Proliferative response to the positive control rIL-1β. The data in (A) and (B) are the mean cell numbers and SD at 72 h. $^{**}p < 0.01$ and $^{*}p < 0.05$ compared with 0 ng/ml rIL-33 or 0 ng/ml rIL-1β by analysis of variance followed by the Dunnett's post hoc tests (A) and by the paired Student's *t*-test (B); $n = 3–4$. (C) Representative flow cytometry analysis of cell division in fibrocytes from PwAA labeled with PKH-26 and incubated for 72 h in the presence or absence of rIL-33 (10 ng/ml). The grey peaks represent successive generations of fibrocytes stimulated with rIL-33 and the peak outlined in back represents cells incubated in medium alone. (D) Representative flow cytometry analysis of PKH-26-labeled fibrocytes from PwAA stained for intracellular type I collagen (COL1) following stimulation with 10 ng/ml of rIL-33 for 72 h. The horizontal line marks fluorescence intensity greater than that observed with the isotype-matched control for the FITC-COL1 monoclonal antibody.

3.3. Stimulation with rIL-33 induced IL-6 production and enhanced IL-8/CXCL8 release in circulating fibrocytes from PwAA

Because rIL-33 has been found to trigger or enhance the release of proinflammatory and profibrotic factors in different cell types [12], circulating fibrocytes were cultured for 12 h in serum-free medium alone or medium containing increasing concentrations of the recombinant protein and the contents of IL-6, IL-8/CXCL8, TGF-β_1 and COL1 in the conditioned media were quantified by ELISA. Stimulation with the highest dose of rIL-33 induced the production of detectable amounts of IL-6 only in fibrocytes from PwAA (Fig. 4). In the same cells, the recombinant protein also significantly increased the release of IL-8/CXCL8 but not that of TGF-β_1 (Fig. 4). The fibrocytes from PwAA spontaneously released relatively high amounts of COL1 in comparison with cells from NAI (Fig. 4), and their stimulation with rIL-33 for 12 h did not significantly affect the production of this collagenous protein (Fig. 4).

3.4. Inhibition of IL-33 signaling via ST2L attenuated the rIL-33-induced responses

The specificity of the response of circulating fibrocytes from PwAA to rIL-33 was confirmed by using an rST2-Fc fusion protein that binds to IL-33 and inhibits cytokine signaling via ST2L. It significantly attenuated the chemotactic activity of rIL-33 in a previous study [28]. Pre-incubation of rIL-33 (10 ng/ml) with increasing concentrations of rST2-Fc fusion protein progressively attenuated the chemotactic and mitogenic activities of the recombinant protein and also inhibited the increased release of IL-8/CXCL8 induced by rIL-33 (Supplemental Fig. 2). The effects of the rST2-Fc fusion protein were significantly different from those observed with a control Fc fusion protein (Supplemental Fig. 2).

4. Discussion

IL-33 has recently emerged as a potential therapeutic target in asthma [11,12]. Increased amounts of immunoreactive IL-33 are present in the peripheral blood [29] and in the bronchoalveolar lavage fluid [30] of asthmatic patients whose bronchial epithelium produces this cytokine at high levels [30]. Excessive release of IL-33 from asthmatic bronchial epithelial cells may occur in response to insults from infectious agents, allergens and pollutants [11] because the chronically inflamed asthmatic epithelium is more susceptible to injury than normal epithelium. Several studies on the function of IL-33 in asthma have previously focused on the ability of the recombinant protein to promote, either alone or in combination with other stimuli, the differentiation and proinflammatory activity of cells usually present in the inflamed mucosa of asthmatic individuals [11,12]. There is no information on the effects of IL-33 on structural alterations that actually render asthmatics

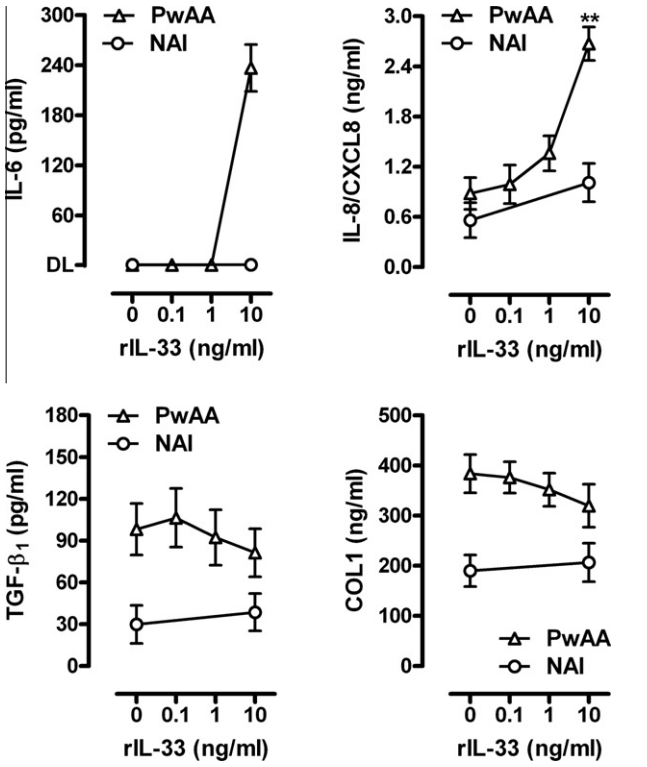

Fig. 4. Production of proinflammatory and profibrotic factors by cultured circulating fibrocytes from patients with allergen-exacerbated asthma (PwAA) and non-asthmatic individuals (NAI). The contents of IL-6, IL-8/CXCL8, TGF-β_1 and soluble type I collagen (COL1) in the conditioned media were measured by ELISA and normalized to cell number. Results are expressed as the means and SD. $^{**}p < 0.01$ compared with 0 ng/ml rIL-33 by analysis of variance followed by the Dunnett's post hoc tests; $n = 4–5$. DL, detection limit of the assay (1.2 pg/ml).

poorly responsive to currently available therapeutic options, such as fibrocyte-mediated subepithelial fibrosis.

This study tested the effects of rIL-33 on the migration and proliferation of circulating fibrocytes because some previous observations have suggested that IL-33 may have direct chemotactic and mitogenic effects in various cell types [23,28,31–33]. The cells were isolated from PwAA because such patients [9] and other individuals whose disease is not adequately controlled by current treatment [8] invariably show increased numbers of fibrocytes in their airways and peripheral blood. The circulating fibrocytes from PwAA exhibited increased levels of expression of ST2L on the cell surface in comparison with the circulating fibrocytes from NAI. Recombinant IL-33 stimulated the directed migration of circulating fibrocytes from PwAA at a concentration (0.1 ng/ml) very close to the concentrations of endogenous IL-33 (120.8 ± 22.4 pg/ml) previously detected in the bronchoalveolar lavage fluid of atopic individuals with moderate asthma [30]. In the circulating fibrocytes from PwAA, rIL-33 also exerted a mitogenic effect in a concentration-dependent manner between 0.1 and 10 ng/ml without causing detectable changes in the release of COL1 in the proliferating cells. In the absence of additional stimuli, a significant effect on the release of proinflammatory cytokines such as IL-6 and IL-8/CXCL8 was observed with concentrations of rIL-33 well above the clinically relevant concentrations.

In conclusion, the results of this study demonstrate that IL-33 exhibits potent chemotactic and mitogenic activities for circulating fibrocytes from PwAA. Therefore, the study uncovers an important mechanism through which fibrocytes may accumulate in the airways of allergic asthmatics when their disease is not adequately controlled by current treatment and provides novel information on the profibrotic function of IL-33 in asthma. The study findings may be relevant to other immunological disorders associated with an increased release of IL-33 and increased recruitment of fibrocytes at tissue sites, particularly systemic sclerosis with interstitial lung involvement [34–37].

Disclosures

S. Mattoli is founding shareholder and board member at AVAIL GmbH. The other authors do not have conflicts of interest or financial interests to disclose.

Author contributions

All authors substantially contributed to the study conception and design, or data acquisition or analysis and interpretation of the results; drafting the manuscript or critically revising the initial draft for important intellectual content; final approval of the submitted version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.047.

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