Increased phenotypic differentiation and reduced corticosteroid sensitivity of fibrocytes in severe asthma

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Background: Patients with severe asthma are less responsive to corticosteroid therapy and show increased airway remodeling. The mesenchymal progenitors, fibrocytes, may be involved in the remodeling of asthmatic airways. We propose that fibrocytes in severe asthma are different from those in nonsevere asthma.
Objectives: To examine the survival, myofibroblastic differentiation, and C-C chemokine receptor 7 (CCR7) expression in blood fibrocytes from patients with severe and nonsevere asthma and study the effect of corticosteroids on fibrocyte function.
Methods: The nonadherent non–T-cell fraction of blood mononuclear cells was isolated from healthy subjects and patients with nonsevere and severe asthma. Total and differentiating fibrocytes were identified by their expression of CD45, collagen I, and α-smooth muscle actin using flow cytometry. The expression of CCR7 and of the glucocorticoid receptor was measured by using flow cytometry.
Results: Increased numbers of circulating fibrocytes, with greater myofibroblastic differentiation potential, were observed in patients with severe asthma. Dexamethasone induced apoptosis, leading to reduction in the number of cultured fibrocytes and total nonadherent non-T cells from healthy subjects and patients with nonsevere asthma but not from patients with severe asthma. Dexamethasone reduced CCR7 expression in fibrocytes from patients with nonsevere asthma but not from patients with severe asthma. Glucocorticoid receptor expression was attenuated in fibrocytes from patients with severe asthma.
Conclusions: Patients with severe asthma have elevated numbers of circulating fibrocytes that show enhanced myofibroblastic differentiation and that are less responsive to the effects of corticosteroids. (J Allergy Clin Immunol 2015;135:1186-95.)

Key words: *Fibrocytes, asthma, corticosteroids, remodeling, myofibroblasts, glucocorticoid receptor, CCR7, nonadherent non-T cells, PBMCs*

The airways in asthma are characterized by airway wall remodeling, including thickening of the airway smooth muscle (ASM) layer and subepithelial fibrosis, which could contribute to airflow obstruction.[1] ASM layer thickening and subepithelial fibrosis in asthma may result from ASM cell hyperplasia and hypertrophy,[2,3] and increased numbers of myofibroblasts[4] in the airway wall. A proportion of asthmatic patients suffer from severe or refractory asthma, which is difficult to control despite receiving high doses of inhaled and sometimes oral corticosteroids.[5] These patients show increased airway wall remodeling, with augmented subepithelial fibrosis and ASM thickening, which may contribute to the persistent airflow obstruction in these patients.[6-8]

Fibrocytes are bone marrow–derived circulating mesenchymal progenitor cells that express not only leukocyte markers such as CD34 and CD45 but also mesenchymal markers such as procollagen and α-smooth muscle actin (α-SMA).[9] Fibrocytes migrate to the lung in response to inflammation, and home on to the airway wall where they differentiate into myofibroblasts. One mediator of fibrocyte migration to the lungs is the C-C chemokine receptor 7 (CCR7).[10,11] CCR7 is expressed on circulating fibrocytes,[12] while the expression of its ligand, chemokine motif ligand-19, is increased in the ASM of patients with asthma, which implicates an important role of CCR7 in the homing of fibrocytes to airways of patients with asthma.[13] Fibrocytes are more abundant in the circulation of subjects with asthma with chronic airflow obstruction, and they have been localized to the ASM compartment in the airway wall of patients with severe refractory asthma.[12,14] At the tissue site, fibrocytes can differentiate into myofibroblasts,[15] which mediate subepithelial fibrosis through the release of extracellular matrix proteins[4] and may also contribute to increased ASM mass.[16]

Corticosteroid insensitivity is a feature of severe asthma. Corticosteroids are less effective in suppressing the release of proinflammatory cytokines from PBMCs[17,18] and alveolar macrophages[19] from patients with severe asthma. ASM cells from patients with severe asthma are also resistant to the antiproliferative and anti-inflammatory effects of corticosteroids.[20,21] Moreover, T lymphocytes from patients with severe refractory asthma and PBMCs exposed to IL-17 and IL-23 are resistant to the induction of cell cycle arrest and apoptosis by corticosteroids.[21-23] However, the effect of corticosteroids on fibrocytes from patients with severe asthma is not known.

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Abbreviations used
ASM: Airway smooth muscle
α-SMA: α-Smooth muscle actin
CCR7: C-C chemokine receptor 7
FITC: Fluorescein isothiocyanate
GR: Glucocorticoid receptor
NANT: Nonadherent non-T
PI: Propidium iodide

We hypothesized that patients with severe asthma have higher numbers of circulating fibrocytes with increased differentiation potential and decreased sensitivity to the effects of corticosteroids. We therefore determined the number of total and differentiating fibrocytes in the nonadherent non-T (NANT)-cell fraction of PBMCs of healthy subjects and patients with nonsevere and severe asthma, immediately after isolation and also after culture. We also compared the expression of the glucocorticoid receptor (GR) in fibrocytes and the effect of dexamethasone on the number of fibrocytes and the expression of CCR7 in these cells. The effect of dexamethasone on apoptosis was also studied in fibrocytes isolated from the adherent fraction of PBMCs.

METHODS

Study population

Healthy subjects and patients with nonsevere and severe asthma were recruited (Table I). Subjects with FEV_1 reversibility of more than 12% or with a PC_{20} of less than 8 mg/mL were diagnosed as asthmatic patients. Severe asthma was defined according to the American Thoracic Society guidelines for refractory asthma, with the presence of at least 1 of 2 major criteria for corticosteroid usage and at least 2 minor criteria.[24] Patients with nonsevere asthma had *controlled asthma* defined as no daytime or nocturnal symptoms, minimal use of rescue medication, and normal lung function while using up to 1000 μg/d or equivalent of inhaled beclomethasone. Current smokers and ex-smokers with a smoking history of more than 5 pack-years were excluded. All patients provided informed consent, and the study was approved by the National Research Ethics Service Committee London - Chelsea (REC 08/H0708/109).

TABLE I. Clinical characteristics of study subjects

Characteristic	Healthy	Nonsevere asthma	Severe asthma
Number	18	18	38
Age (y)	40.2 ± 2.3	44.6 ± 3.7	49.2 ± 2.4
Sex: female/male	8/10	10/8	28/10
Duration of asthma (y)	NA	29.0 ± 11.5	28.8 ± 15.7*
Smoking status	None	None	None
Inhaled corticosteroid dose (μg BDP equivalent)	0	392.3 ± 624.4	1617 ± 537.6†‡
Atopy	10	11	27
Receiving oral corticosteroids	0	0	26
Prebronchodilator FEV_1 (L)	3.5 ± 0.2	2.8 ± 0.2†	1.9 ± 0.1§‖
Prebronchodilator FEV_1 of predicted value (%)	97.9 ± 2.9	87.1 ± 3.1†	67.9 ± 3.5§‡
FEV_1/FVC (%)	87.4 ± 1.6	73.4 ± 1.8§	69.4 ± 1.9§

Data are expressed as mean ± SEM.
BDP, Beclomethasone dipropionate; *FVC*, forced vital capacity; *ICS*, inhaled corticosteroids; *NA*, not applicable/available.
*$P < .05$ compared with healthy subjects.
†$P < .01$ compared with healthy subjects.
‡$P < .01$ compared with patients with nonsevere asthma.
§$P < .001$ compared with healthy subjects.
‖$P < .05$ compared to patients with nonsevere asthma.

Isolation of circulating fibrocytes from the NANT-cell fraction

NANT cells were isolated from peripheral blood as previously described.[12] PBMCs were isolated from whole blood using density gradient centrifugation with Ficoll-Paque PLUS (GE HealthCare, Uppsala, Sweden). Adherent cells were removed by adherence, and the nonadherent cell fraction was depleted of T cells using magnetic beads coated with anti-CD3 mAb (Miltenyi Biotec, Auburn, Calif). The resulting NANT cells were cultured in Iscove modified Dulbecco medium (Sigma-Aldrich, Ayrshire, United Kingdom) containing 30% FBS and 1% BSA at 37°C and 5% CO_2 for the required time periods in the presence or absence of the required treatments. The number of viable NANT cells was determined by Trypan blue staining and hemocytometer counting.

Fibrocytes were identified in freshly isolated or cultured NANT cells as $CD45^+$/collagen I^+ cells, while differentiating fibrocytes as α-SMA^+ cells, using flow cytometry. NANT cells were fixed using 0.5% paraformaldehyde, washed twice with PBS, and permeabilized using BD FACS Permeabilizing Solution 2 (BD Biosciences, San Jose, Calif). Cell pellets were incubated with mouse anti-human collagen I antibody (Millipore Corporation, Temecula, Calif) followed by a fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibody (DAKO A/S, Glostrup, Denmark). Cell pellets were then incubated with an allophycocyanin-conjugated mouse-anti-human CD45 antibody (BD Biosciences). In some experiments, cells were also stained using a phycoerythrin-conjugated mouse anti-human CCR7 antibody (BD Biosciences). Stained cells were analyzed using a BD FACSCanto II flow cytometer. The relevant isotype antibody controls were used for every flow cytometry experiment.

Isolation of fibrocytes from adherent PBMCs

In some experiments, fibrocytes isolated from the adherent fraction of PBMCs were used. Briefly, PBMCs were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% FBS for 3 days. Nonadherent cells were then removed, and adherent cells were cultured for a further 3 days in the presence of the required treatments. Viable cells were counted, and the percentage of fibrocytes was determined as described above.

Determination of GR expression in fibrocytes

NANT cells were fixed, stained with an allophycocyanin-conjugated mouse anti-human CD45 antibody (BD Biosciences), and permeabilized as described above. Cell pellets were then incubated with a FITC-conjugated mouse anti-human collagen I antibody (Millipore), and a rabbit-anti-human GR antibody (Abcam, Mass) followed by a phycoerythrin-conjugated goat-anti-rabbit antibody (Abcam). Stained cells were analyzed by using flow cytometry as described above.

Determination of apoptotic NANT cells using Annexin V and propidium iodide staining

Apoptosis of NANT cells was analyzed by Annexin V and propidium iodide (PI) staining using the FITC Annexin V/Dead cell apoptosis kit (Invitrogen, Paisley, United Kingdom) according to the manufacturer's instructions. Briefly, NANT cells were treated as required, washed in PBS and then incubated with FITC-conjugated Annexin V and PI, and analyzed by using flow cytometry. Annexin V^-/PI^- were considered live cells, Annexin V^+/PI^- as apoptotic, and Annexin V^+/PI^+ as late apoptotic cells.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was carried out using the GraphPad Prism (version 5) software package (GraphPad Prism

Software, Inc, San Diego, Calif). Results were analyzed using the Friedman test for intragroup comparisons, and the Kruskal-Wallis test for comparisons between healthy and nonsevere and severe asthmatic groups, followed by Dunns posttest. Correlations between parameters were determined by Spearman rank correlation. $P < .05$ was considered statistically significant.

RESULTS

Increased circulating fibrocytes in patients with severe asthma

Immediately after isolation there was no significant difference in the number of NANT cells per milliliter of blood between the 3 groups (see Fig E1 in this article's Online Repository at www.jacionline.org). However, the NANT cells of patients with severe asthma contained a higher percentage of fibrocytes than did those of patients with nonsevere asthma and healthy subjects, whereas the percentage of circulating fibrocytes in patients with nonsevere asthma was higher than that in healthy subjects (Fig 1, *A* and *B*). These differences were thus reflected in the absolute number of fibrocytes per milliliter of blood, which was increased in those with severe asthma compared with those with nonsevere asthma and healthy subjects and also in those with nonsevere asthma compared with healthy subjects (Fig 1, *C*). The number of circulating fibrocytes in those with severe asthma who were on oral prednisolone was not significantly different from that of patients who were not (see Fig E2 in this article's Online Repository at www.jacionline.org), suggesting that systemic corticosteroid therapy did not affect the number

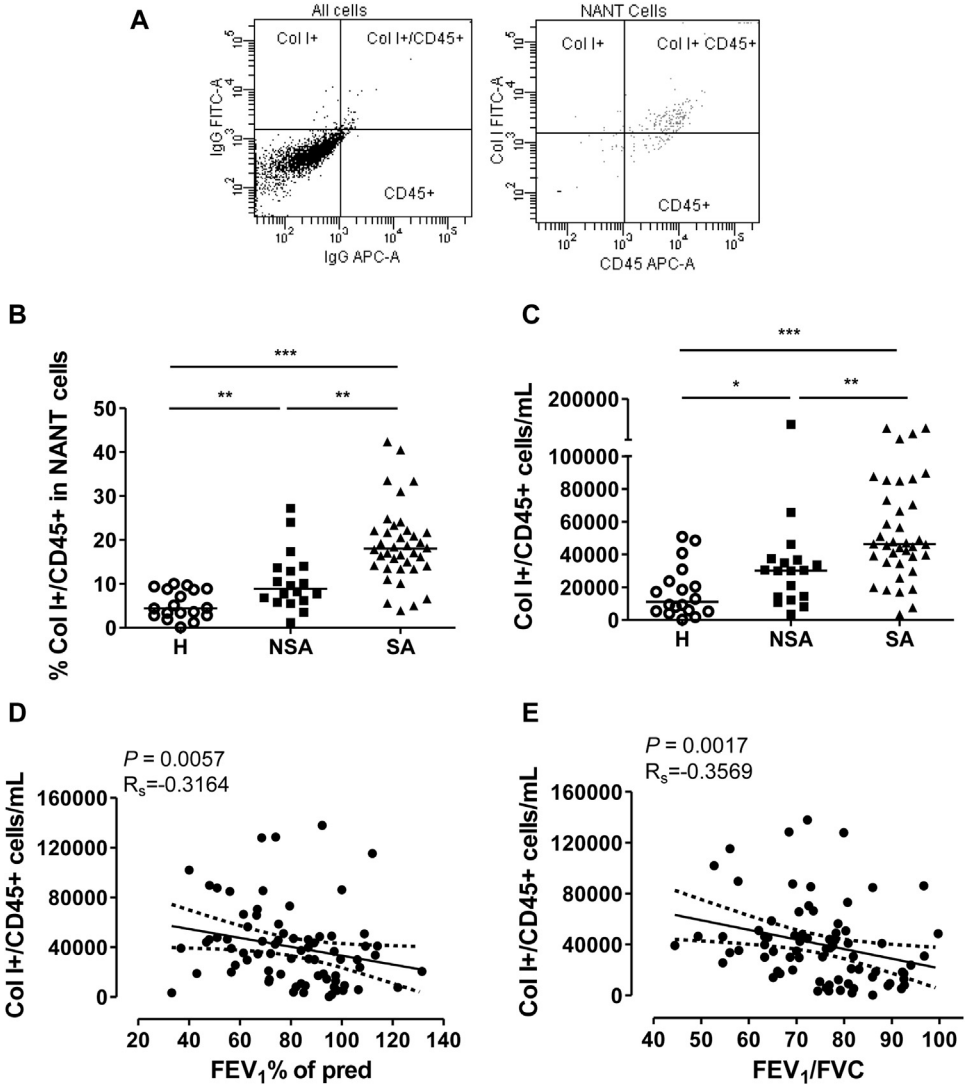


FIG 1. Number of circulating fibrocytes in freshly isolated NANT cells from healthy subjects and patients with nonsevere and severe asthma. Representative flow cytometry scatter plots from 1 experiment **(A)**. The percentage **(B)** and number **(C)** of fibrocytes (Col I^+/$CD45^+$ cells) in freshly isolated NANT cells from healthy subjects (*H*; n = 18) and patients with nonsevere asthma (*NSA*; n = 18) and severe asthma (*SA*; n = 38) was determined. *Horizontal lines* represent the median for each group. **D** and **E,** Correlation of fibrocyte numbers with FEV_1 % predicted (Fig 1, *D*) and FEV_1/FVC ratio (Fig 1, *E*) was determined using Spearman rank correlation. *Col I*, Collagen I; *FVC*, forced vital capacity; R_S, Spearman rank correlation. $*P < .05$, $**P < .01$, and $***P < .001$.

of circulating fibrocytes. Moreover, the number of fibrocytes per milliliter of blood showed an inverse correlation with the prebronchodilator FEV_1% (Fig 1, *D*) and FEV_1/forced vital capacity ratio (Fig 1, *E*).

Increased differentiation of fibrocytes from patients with severe asthma

We observed an increase in the number of fibrocytes in the NANT cells from healthy subjects and patients with nonsevere asthma after culture, peaking after 3 days in culture, whereas the number of fibrocytes from patients with severe asthma did not increase (Fig 2, *A*). We used single α-SMA staining to identify differentiating fibrocytes because we have established that the majority cells in the NANT-cell population expressing α-SMA are fibrocytes (data not shown). The number of differentiating fibrocytes in the NANT cells of all groups increased with time, peaking after 3 days (Fig 2, *B*). Accumulation of α-SMA–positive cells was followed by an increase in adherent "spindle-shaped" cells, which peaked after 10 days, indicating that fibrocytes can fully differentiate into myofibroblasts-like cells (see Fig E3 in this article's Online Repository at www.jacionline.org). Intriguingly, the number of differentiating fibrocytes in the NANT cells from patients with severe asthma was higher than the number in healthy subjects and patients with nonsevere asthma at day 3 (Fig 2, *B*).

Effect of corticosteroids on apoptosis of NANT cells and fibrocytes

Dexamethasone (10^{-8}-10^{-6} mol/L) treatment for 3 days led to a concentration-dependent reduction in the number of fibrocytes and differentiating fibrocytes from healthy subjects and patients with nonsevere asthma but had no effect on those from patients with severe asthma (Fig 3, *A* and *B*). Dexamethasone (10^{-8}-10^{-6} mol/L) also reduced the number of total NANT cells from healthy subjects and patients with nonsevere asthma but not those from patients with severe asthma (Fig 3, *C*).

We determined the percentage of apoptotic NANT cells both at baseline and after dexamethasone treatment. Immediately after isolation, the percentage of live and apoptotic cells was similar in all groups (Fig 3, *D*). After 3 days in culture, untreated NANT cells from patients with severe asthma contained a higher percentage of live cells and a lower percentage of early apoptotic cells than did NANT cells from healthy subjects, suggesting increased survival of NANT cells in those with severe asthma (Fig 3, *E*). Dexamethasone caused a reduction in the percentage of live cells while increasing the percentage of early and late apoptotic cells in NANT cells from healthy subjects (Fig 3, *F*) and patients with nonsevere asthma (Fig 3, *G*). However, dexamethasone did not modulate the percentage of live and apoptotic NANT cells from patients with severe asthma (Fig 3, *H*). Similarly, when the data were expressed as a total percentage of apoptotic cells, NANT cells from patients with severe asthma showed a higher percentage of live, and a lower percentage of apoptotic, cells than did those from healthy subjects (see Table E1 in this article's Online Repository at www.jacionline.org). Moreover, dexamethasone caused a significant decrease in the percentage of total apoptotic cells from healthy subjects and patients with nonsevere asthma but had no effect on those from patients with severe asthma (see Table E1).

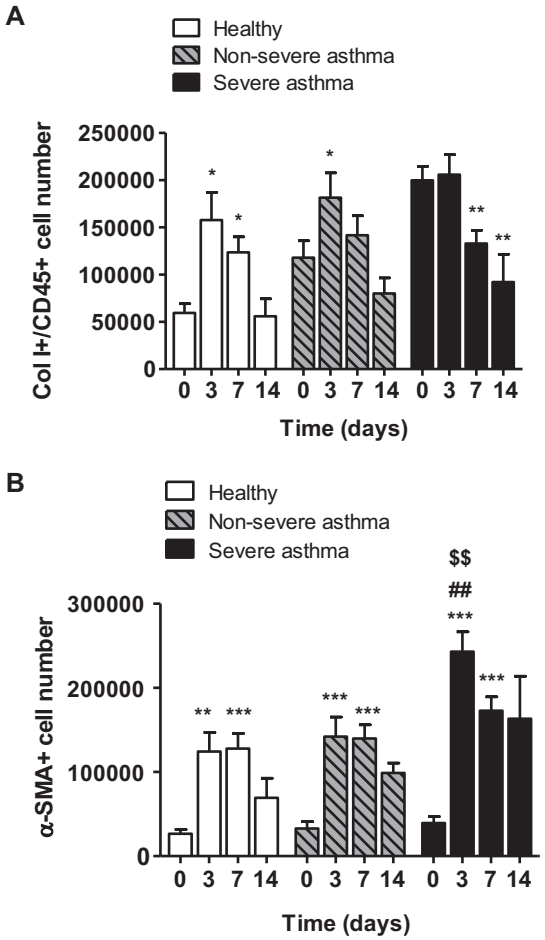


FIG 2. Number of fibrocytes and differentiating fibrocytes in cultured NANT cells from healthy subjects and patients with nonsevere and severe asthma. The number of fibrocytes (Col I^+/CD45+ cells; **A**) and differentiated fibrocytes (α-SMA^+ cells; **B**) were determined in NANT cells from healthy subjects (n = 5-18) and patients with nonsevere (n = 4-18) and severe asthma (n = 5-38) after 3 to 14 days in culture. *Col I*, Collagen I. *Bars* represent mean ± SEM. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$ versus day 0 for each patient group. $^{\#\#}P < .01$ versus healthy day 3. $^{\$\$}P < .01$ versus nonsevere asthma day 3.

Dexamethasone (10^{-7} mol/L)-induced reduction in NANT-cell (Fig 4, *A*), fibrocyte (Fig 4, *B*), and differentiating fibrocyte (Fig 4, *C*) numbers was prevented by the GR antagonist RU486 (10^{-6}-10^{-5} mol/L), confirming that this effect is mediated by GR.

Corticosteroid insensitivity of fibrocytes on CCR7 expression in severe asthma

No significant difference was observed in the baseline percentage of CCR7-positive fibrocytes between the 3 groups (Fig 5, *A* and *B*). However, dexamethasone (10^{-8}-10^{-6} mol/L) treatment for 3 days led to a concentration-dependent decrease in the percentage of CCR7-positive fibrocytes (Fig 5, *A* and *C*) and the CCR7 median fluorescence intensity ratio (Fig 5, *D*) in NANT cells from patients with nonsevere asthma but had no significant effect on those from patients with severe asthma. Thus, the expression of CCR7 on the fibrocytes of patients with severe asthma is resistant to the inhibitory effect of dexamethasone.

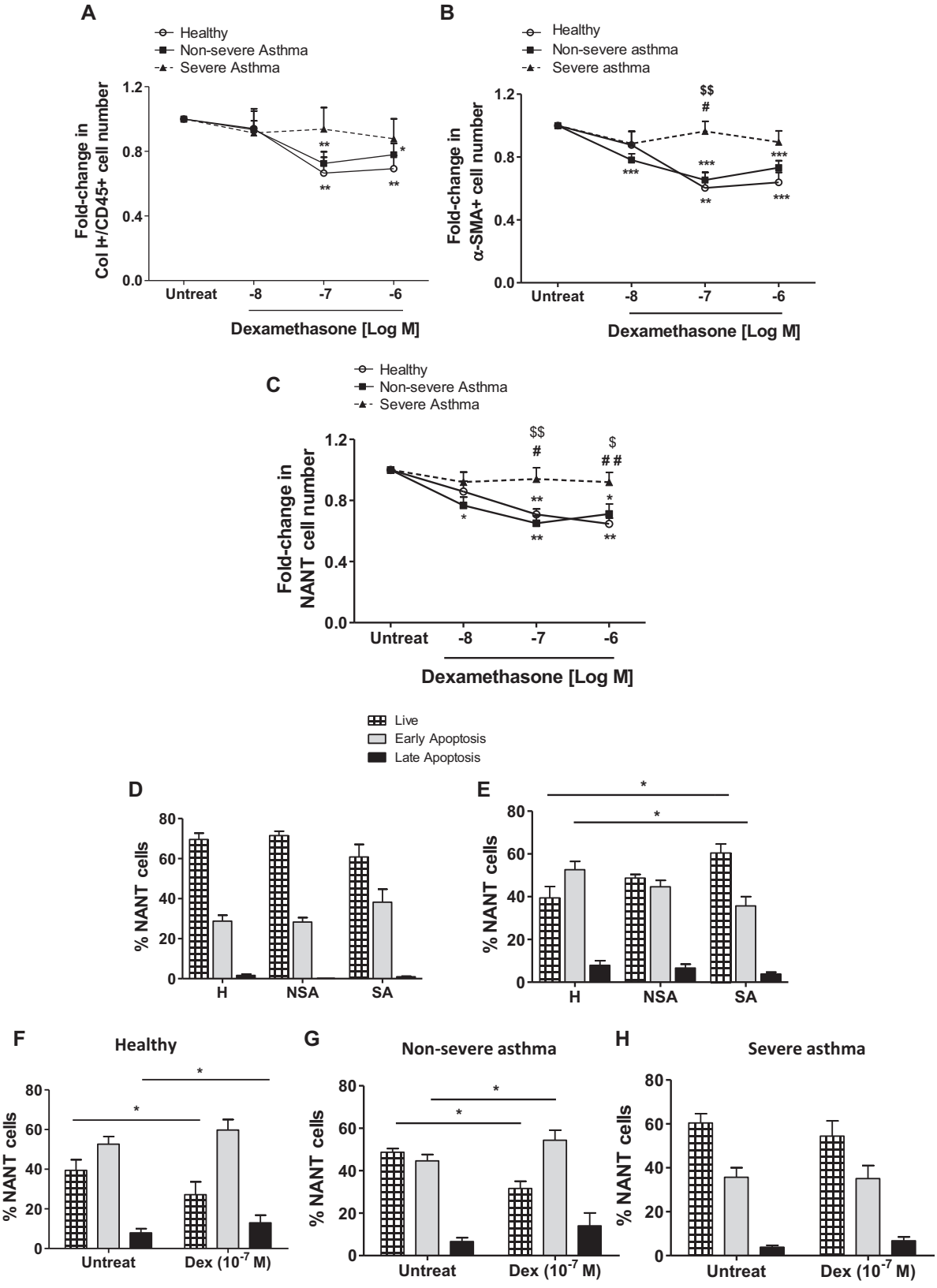


FIG 3. Effect of dexamethasone on fibrocyte and differentiating fibrocyte number. **A-C,** NANT cells from healthy subjects (n = 9) and patients with nonsevere (n = 10) and severe (n = 12) asthma were cultured for 3 days in the presence or absence of dexamethasone (10^{-8}-10^{-6} mol/L). The number of fibrocytes (Col I^+/$CD45^+$ cells; Fig 3, *A*), differentiating fibrocytes (α-SMA^+ cells; Fig 3, *B*), and total NANT cells (Fig 3, *C*) was determined. Data are expressed as fold change compared with untreated control. **D** and **E,** The

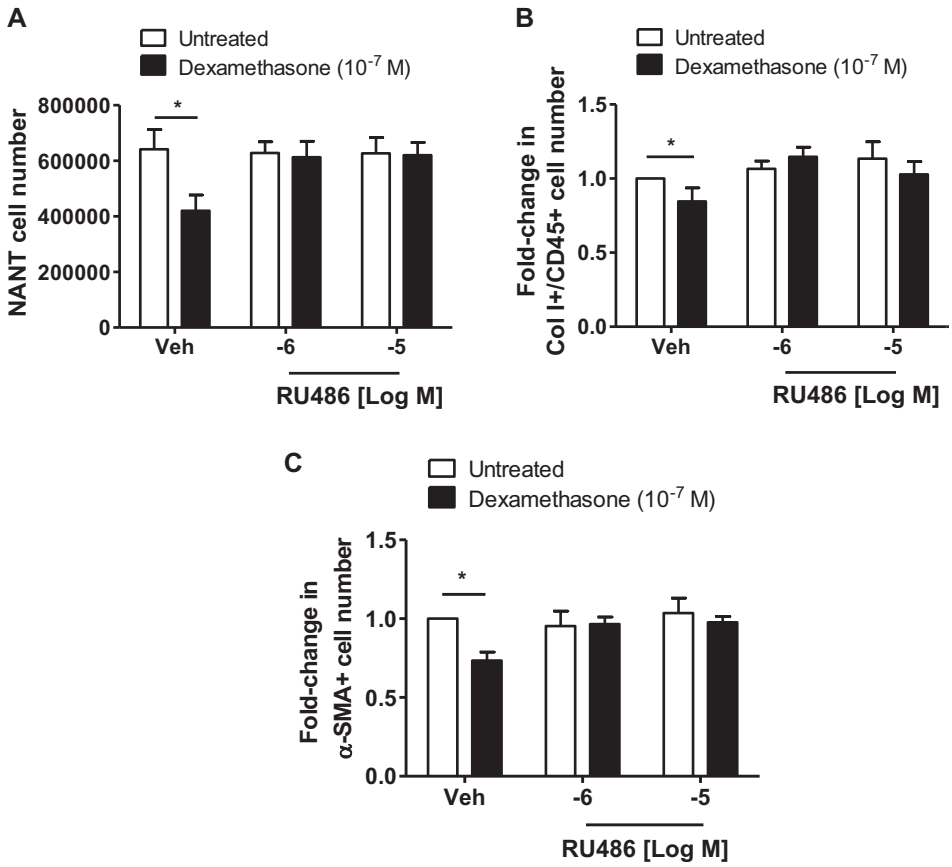


FIG 4. Effect of GR inhibition on dexamethasone-induced reduction in fibrocyte number. NANT cells from healthy subjects were treated with dexamethasone (10^{-7} mol/L) in the presence or absence of RU486 (10^{-6}-10^{-5} mol/L) and total NANT-cell **(A)**, fibrocyte (Col I^+/$CD45^+$ cells; **B**) and differentiating fibrocyte (α-SMA^+ cells; **C**) numbers were determined after 3 days. *Bars* represent mean ± SEM of 6 donors. *Col I*, Collagen I. *$P < .05$.

GR expression in fibrocytes in severe asthma

We compared the expression of GR in fibrocytes in freshly isolated NANT cells from healthy subjects and patients with nonsevere and severe asthma (Fig 6, *A*). Both the percentage of GR-positive fibrocytes (Fig 6, *B*) and the GR median fluorescence intensity ratio (Fig 6, *C*) were lower in patients with severe asthma than in healthy subjects and patients with nonsevere asthma. The percentage of GR-positive cells and GR median fluorescence intensity ratio were also reduced in the whole NANT cell isolated from patients with severe asthma compared with healthy subjects and patients with nonsevere asthma (see Fig E4 in this article's Online Repository at www.jacionline.org).

Effect of dexamethasone on fibrocytes isolated from adherent PBMCs

The percentage of fibrocytes and differentiating fibrocytes in adherent PBMCs increased in a time-dependent manner, peaking after 10 days in culture (see Fig E5 in this article's Online Repository at www.jacionline.org). Treatment with dexamethasone (10^{-7} mol/L) for 72 hours led to a decrease in the number of fibrocytes and differentiating fibrocytes from healthy subjects, and this effect was prevented by RU486 (10^{-6} mol/L) (Fig 7, *A* and *B*). Dexamethasone (10^{-7} mol/L) also reduced the number of fibrocytes and differentiating fibrocytes from patients with nonsevere asthma but had no significant effect on those from patients with severe asthma (Fig 7, *C* and *D*). Thus, fibrocytes isolated from the adherent fraction of PBMCs of patients with severe asthma are also resistant to corticosteroid-induced apoptosis.

DISCUSSION

We have demonstrated that immediately after isolation, there is a higher number of fibrocytes in the circulating blood of patients with severe asthma than in the blood of healthy subjects and patients with nonsevere asthma. Furthermore, we showed that when placed in culture, fibrocytes from patients with severe asthma have a greater capacity to differentiate into myofibroblasts

percentage of live and early and late apoptotic NANT cells from healthy subjects (*H*; n = 6) and patients with nonsevere asthma (*NSA*; n = 6) and severe asthma (*SA*; n = 6) was determined immediately after isolation (Fig 3, *D*), or after 3 days in culture in the absence (Fig 3, *E*) or presence of dexamethasone (10^{-7} mol/L; **F-H**). *Col I*, Collagen I. Data points and bars represent mean ± SEM. *$P < .05$, **$P < .01$, ***$P < .001$ versus untreated for each group. $^{\#}P < .05$, $^{\#\#}P < .01$ versus healthy. $^{\$}P < .05$, $^{\$\$}P < .01$ versus nonsevere asthma.

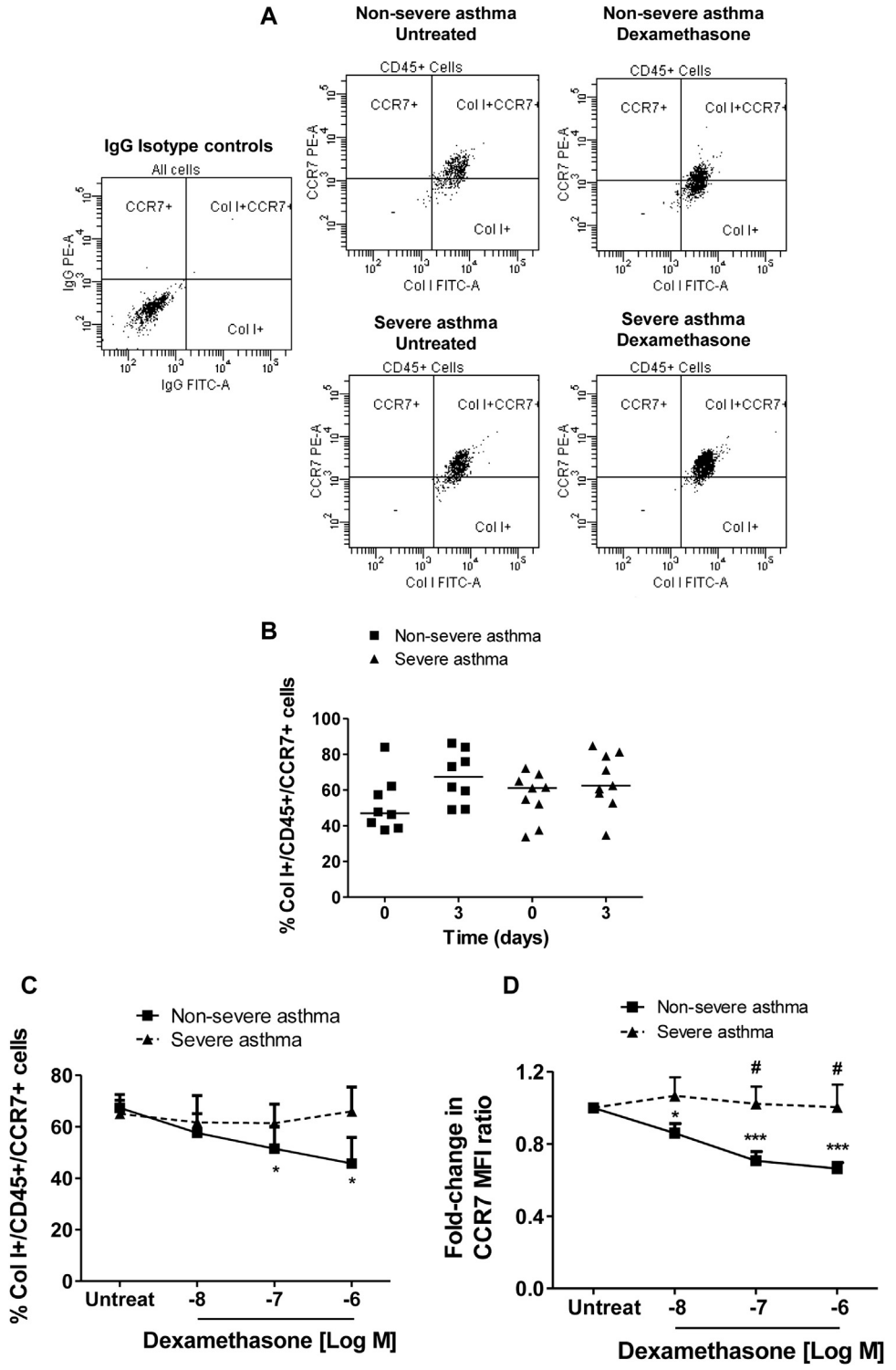


FIG 5. Effect of dexamethasone on CCR7 expression in fibrocytes from patients with nonsevere and severe asthma. **A,** Representative flow cytometry scatter plots from 1 experiment per group. **B,** The percentage of $CCR7^+$ fibrocytes ($Col\ I^+/CD45^+/CCR7^+$ cells) was determined in NANT cells from patients with nonsevere asthma (*NSA*; n = 10) and severe asthma (*SA*; n = 12) immediately after isolation or after 3 days in culture. *Horizontal lines* represent the median for each group. **C** and **D,** The percentage of $CCR7^+$ fibrocytes (Fig 5, *C*) and the median fluorescence intensity (*MFI*) ratio (Fig 5, *D*) after treatment with dexamethasone (10^{-7} mol/L) for 3 days was determined in the NANT cells of the same patients. *Col I*, Collagen I. Data points represent mean ± SEM. $^*P < .05$, $^{***}P < .001$ versus untreated. $^{\#}P < .01$ versus nonsevere asthma.

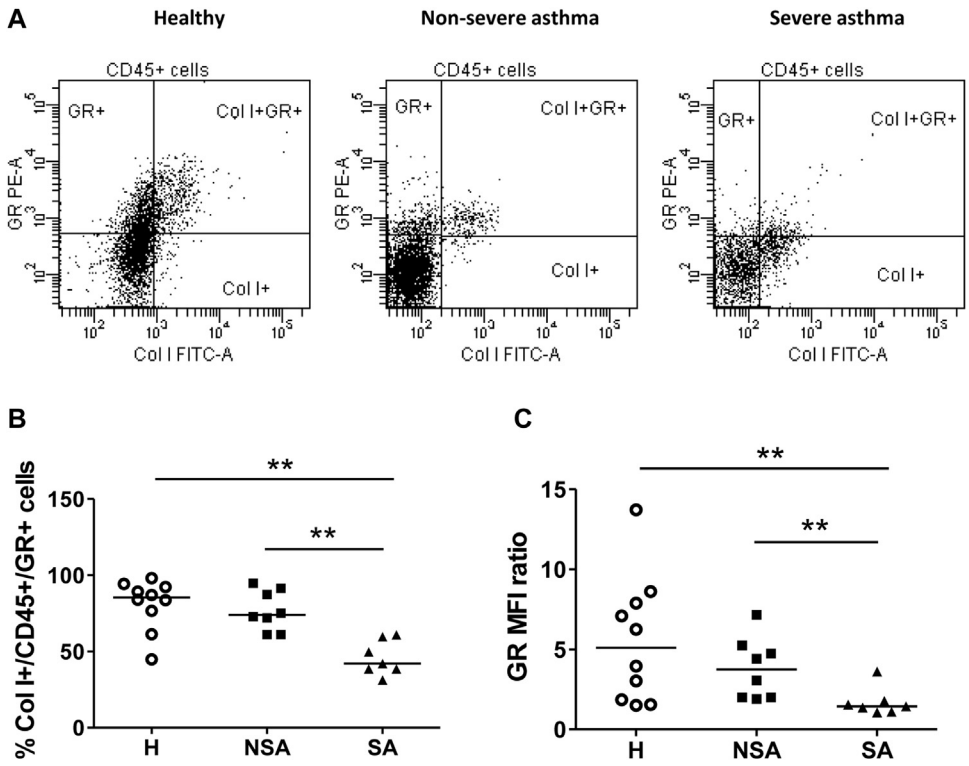


FIG 6. GR expression in fibrocytes from healthy subjects and patients with nonsevere and severe asthma. **A,** Representative flow cytometry scatter plots from 1 experiment per group. The percentage **(B)** and median fluorescence intensity (*MFI*) ratios **(C)** of GR^+ fibrocytes ($Col\ I^+/CD45^+/GR^+$ cells) were determined in freshly isolated NANT cells from healthy subjects (*H*; n = 10) and patients with nonsevere asthma (*NSA*; n = 8) and severe asthma (*SA*; n = 7). *Col I*, Collagen I. *Horizontal lines* represent medians. $^{**}P < .01$.

than do fibrocytes from healthy subjects and patients with nonsevere asthma. Dexamethasone decreased the number of NANT cells, including fibrocytes, from healthy subjects and patients with nonsevere asthma but had no effect on those from patients with severe asthma. In line with these findings, dexamethasone reduced the percentage of live NANT cells and increased the percentage of apoptotic NANT cells from healthy subjects and patients with nonsevere asthma but had no effect on those from patients with severe asthma. Fibrocytes from patients with severe asthma were also resistant to the inhibition of CCR7 expression by dexamethasone and showed reduced GR expression than did those from healthy subjects and patients with nonsevere asthma. Thus, patients with severe asthma have a higher number of circulating fibrocytes that have a greater capacity to differentiate into myofibroblasts while showing relative corticosteroid insensitivity in terms of both apoptotic response and suppression of CCR7 expression.

The airway wall in severe asthma is characterized by increased ASM thickening and subepithelial fibrosis, features of a remodeling process.[6-8] We show that fibrocytes are more abundant in the circulation of patients with severe asthma and have an increased capacity to differentiate into myofibroblasts than in the circulation of healthy subjects and patients with nonsevere asthma. Our data are in line with findings by Saunders et al[14] showing increased numbers of α-SMA–positive fibrocytes in the lamina propria of patients with severe refractory asthma and in the ASM bundles of patients with asthma of all severities, suggesting that fibrocytes can migrate to the airway wall of these patients and differentiate into myofibroblasts.[14] Myofibroblasts exhibit a phenotype between fibroblasts and smooth muscle cells that involves not only secretion of extracellular matrix proteins[25] but also expression of smooth muscle–specific proteins.[26] The number of myofibroblasts in the airways of patients with asthma was found to be correlated to the extent of subepithelial fibrosis.[4] Myofibroblasts have also been detected in the ASM bundles of patients with asthma, thereby possibly contributing to the increased ASM mass.[16] Thus, patients with severe asthma may have a greater pool of circulating fibrocytes that can readily differentiate into myofibroblasts on migration to the airway wall, contributing to the remodeling process.

Patients with severe asthma show exaggerated airway remodeling despite being on high doses of inhaled and sometimes oral corticosteroid treatment.[6-8] We, therefore, determined the effect of corticosteroids on the function of fibrocytes isolated from these patients. Our data show a reduction in the number of fibrocytes and differentiating fibrocytes from healthy subjects and patients with nonsevere asthma in response to exposure to dexamethasone, which was prevented by the GR antagonist, RU486. Moreover, dexamethasone reduced the number of total NANT cells while increasing the percentage of apoptotic NANT cells, implicating GR-mediated apoptosis in this effect. Hayashi et al[27] have also recently reported dexamethasone-induced inhibition of the proliferation of circulating fibrocytes of healthy subjects.[27] Dexamethasone also led to a reduction in the expression of CCR7 in the fibrocytes from patients with nonsevere asthma, in line with reports in dendritic cells,[28,29] suggesting that corticosteroids may have an inhibitory effect on the migration of fibrocytes.

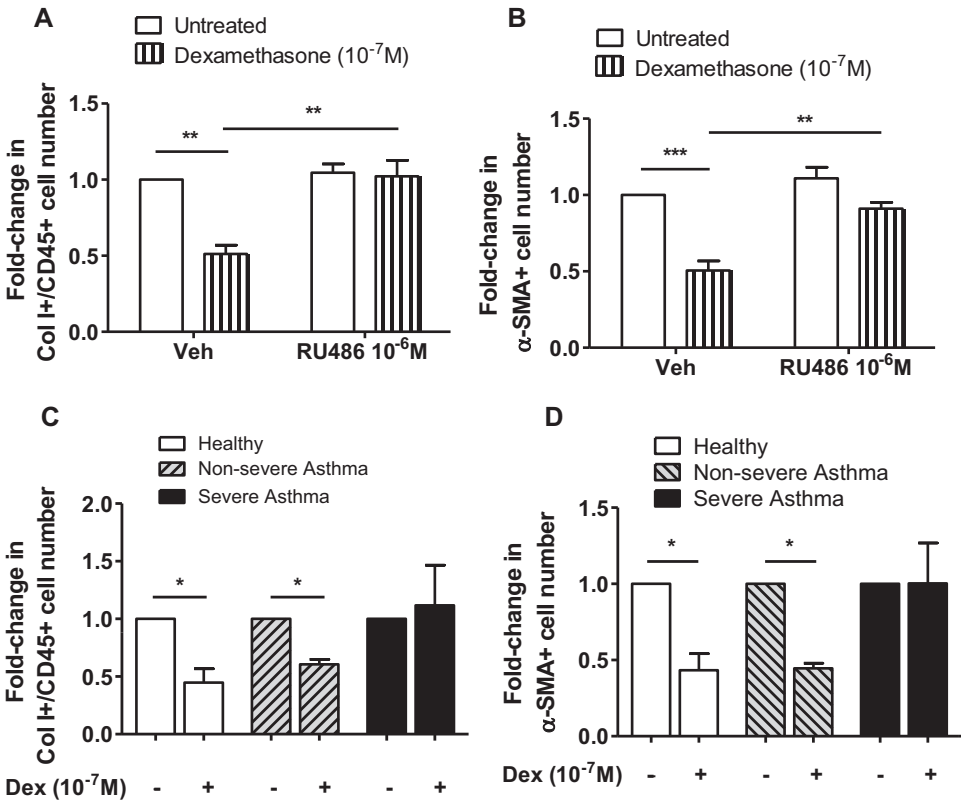


FIG 7. Effect of dexamethasone on the number of fibrocytes derived from adherent PBMCs. **A** and **B,** Adherent PBMCs from healthy subjects (n = 6) were placed in culture for 3 days followed by incubation with dexamethasone (10^{-7} mol/L), in the presence or absence of RU486 (10^{-6} mol/L), for a further 3 days. The number of fibrocytes (Col I^+/$CD45^+$ cells; Fig 7, *A*) and differentiating fibrocytes (α-SMA+ cells; Fig 7, *B*) was determined. **C** and **D,** Adherent PBMCs from healthy subjects (n = 6) and patients with nonsevere (n = 5) and severe (n = 6) asthma were placed in culture for 3 days followed by incubation with dexamethasone (10^{-7} mol/L) for a further 3 days. The number of fibrocytes (Col I^+/$CD45^+$ cells; Fig 7, *C*) and differentiating fibrocytes (α-SMA^+ cells; Fig 7, *D*) was determined. *Col I*, Collagen I. *Bars* represent mean ± SEM. **P* < .05, ** *P* < .01, and ****P* < .001.

Intriguingly, dexamethasone had no effect on both the number or the expression of CCR7 in fibrocytes from patients with severe asthma, indicating that these cells show relative corticosteroid insensitivity. This may result from the lower GR expression levels we observed in fibrocytes from those with severe asthma. Changes in the relative expression of GRα and GRβ have been implicated in corticosteroid insensitivity in PBMCs and macrophages of patients with asthma[23,30]; however, other studies have reported that this is not the case.[31-34] The antibody used in our study does not distinguish between the 2 GR isoforms, and so we were unable to explore this potential mechanism. Other mechanisms such as reduced histone deacetylase activity[17] and p38 mitogen-activated protein kinase–dependent GR phosphorylation[18-20,35] may also be involved. Dexamethasone also failed to induce apoptosis and reduce the number of total NANT cells from patients with severe asthma. NANT cells from patients with severe asthma also exhibited lower levels of GR expression than did those from healthy subjects and patients with nonsevere asthma. Thus, the relative corticosteroid insensitivity we observed in fibrocytes from patients with severe asthma also extends to nonfibrocyte cells. Nonetheless, because we have shown that NANT cells from patients with severe asthma show increased survival, we cannot exclude the possibility that the failure of dexamethasone to reduce the number of these cells is a result of an inherent resistance to apoptosis.

We have previously demonstrated relative corticosteroid insensitivity in PBMCs,[17] alveolar macrophages,[19] and ASM cells[20] from patients with severe asthma. We now extend this observation to show that in patients with severe asthma, circulating progenitor cells such as fibrocytes may also exhibit an altered response to corticosteroids. This finding may have important implications in understanding the mechanisms underlying the increased remodeling observed in these patients.

The most commonly described method of isolating fibrocytes from blood uses the adherent fraction of PBMCs.[36,37] However, fibrocytes are also abundant in the nonadherent fraction of PBMCs.[12,38] We found that fibrocytes isolated from the adherent fraction of PBMCs of patients with severe asthma were also resistant to the proapoptotic effect of dexamethasone, suggesting that both adherent and nonadherent fibrocyte populations show the same pattern of response to corticosteroids *in vitro*.

Although we show that systemic corticosteroid use does not affect circulating fibrocyte numbers, we cannot exclude the possibility that systemic corticosteroids could be the cause of some of the other phenotypic differences we observed in fibrocytes of patients with severe asthma. Another limitation of our study is

that it does not provide direct evidence for the role of fibrocyte-derived myofibroblasts in the development of airway remodeling in severe asthma. A study involving tracking fluorescence-labeled fibrocytes in a mouse model of allergic asthma has shown recruitment of fibrocytes from the circulation to the bronchial tissue and differentiation into myofibroblasts in response to allergen exposure.[15] Similar studies in animal models of severe asthma may provide a clearer picture of the involvement of fibrocytes in the development of airway remodeling in this disease.

Fibrocytes are potentially important cells involved in the airway wall remodeling in asthma.[10] Our study demonstrates that patients with severe asthma have elevated numbers of circulating fibrocytes, which show an aberrant phenotype involving a heightened ability to differentiate into myofibroblasts and relative resistance to the effects of corticosteroids. Fibrocytes could thus play a central role in the development and persistence of airway remodeling in severe asthma and could be an important therapeutic target.

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Key messages

- **Patients with severe asthma have a higher number of circulating fibrocytes that have a higher capacity to undergo myofibroblastic differentiation in culture.**
- **Fibrocytes of patients with severe asthma are more resistant to the induction of apoptosis and to the inhibition of CCR7 expression by corticosteroids.**

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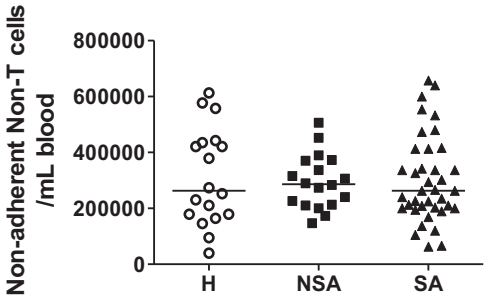


FIG E1. Number of freshly isolated NANT cells from healthy subjects and patients with nonsevere and severe asthma. The number of freshly isolated NANT cells from healthy subjects (*H*; n = 18) and patients with nonsevere asthma (*NSA*; n = 18) and severe asthma (*SA*; n = 38) was determined. *Horizontal lines* represent the median for each group.

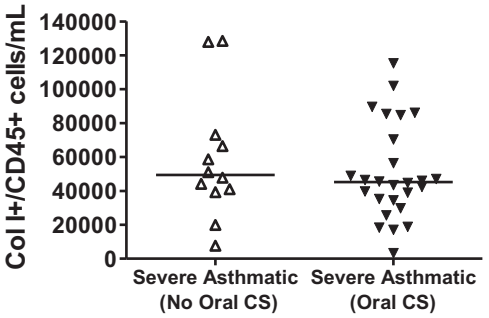


FIG E2. Number of fibrocytes in freshly isolated NANT cells from patients with severe asthma on oral corticosteroid (*CS*) treatment. The number of fibrocytes (Col I^+/$CD45^+$ cells) in freshly isolated NANT cells from patients with severe asthma on oral corticosteroids (n = 26) or not (n = 12) was determined. *Col I*, Collagen I. *Horizontal lines* represent the median for each group.

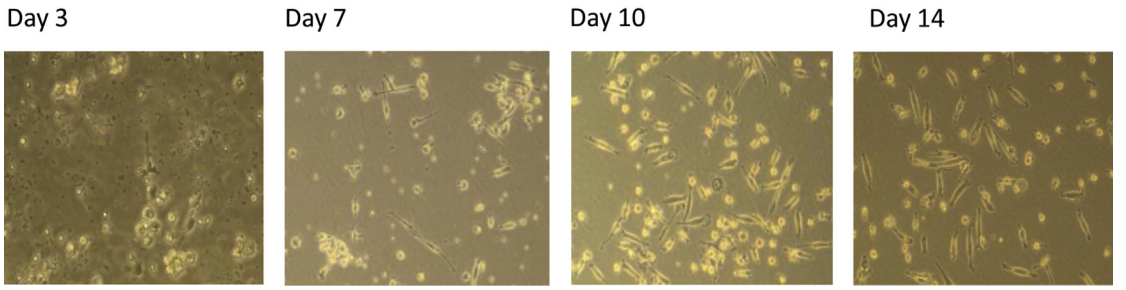


FIG E3. Appearance of myofibroblast-like cells in cultured NANT cells. NANT cells were placed in culture for a period of 3 to 14 days and the appearance of myofibroblast-like, spindle-shaped cells was observed under a light microscope. Representative pictures are shown.

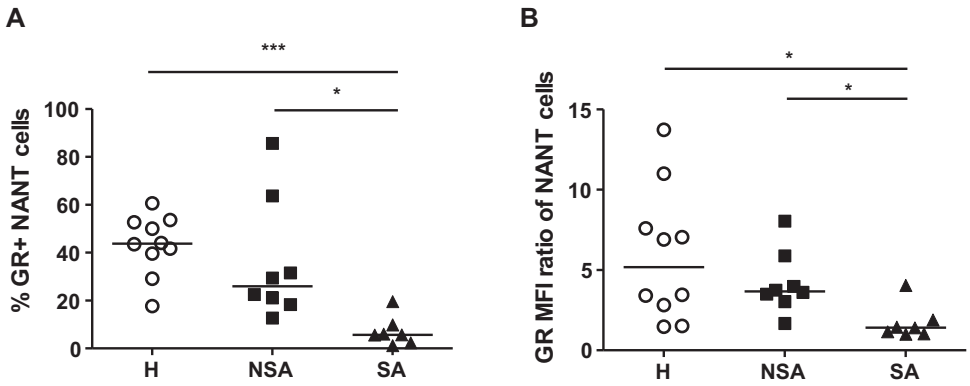


FIG E4. GR expression in NANT cells from healthy subjects and patients with nonsevere and severe asthma. The percentage **(A)** and median fluorescence intensity (*MFI*) ratios **(B)** of GR^+ NANT cells were determined in freshly isolated NANT cells from healthy subjects (*H*; n = 10) and patients with nonsevere asthma (*NSA*; n = 8) and severe asthma (*SA*; n = 7). *Horizontal lines* represent the median for each group. $*P < .05$ and $***P < .001$.

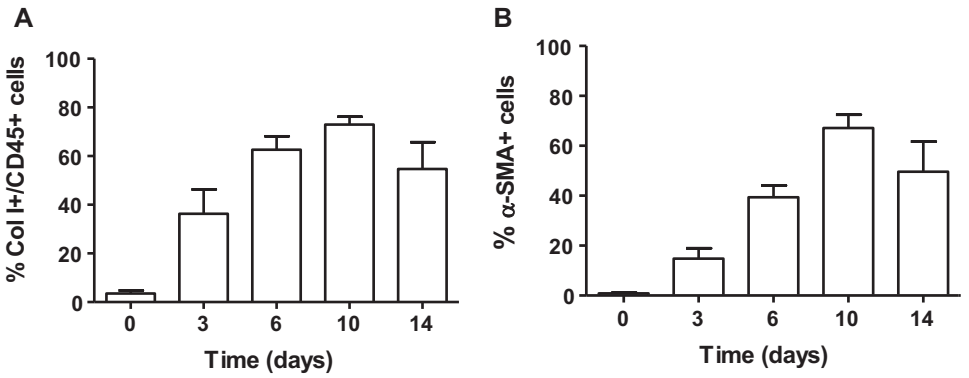


FIG E5. Percentage of fibrocytes and differentiating fibrocytes in cultured adherent PBMCs. The number of fibrocytes (Col I^+/$CD45^+$ cells; **A**) and differentiating fibrocytes (α-SMA^+ cells; **B**) was determined in adherent PBMCs from healthy subjects (n = 3) immediately after isolation (day 0) and after culture for 3 to 14 days. *Col I*, Collagen I. *Bars* represent mean ± SEM.

TABLE E1. Effect of dexamethasone on the percentage of live and apoptotic NANT cells from healthy subjects and patients with nonsevere and severe asthma

	Healthy (%)		Nonsevere asthma (%)		Severe asthma (%)	
Experimental condition	**Live**	**Apoptotic**	**Live**	**Apoptotic**	**Live**	**Apoptotic**
Untreated	39.43 ± 5.3	60.55 ± 5.3	48.72 ± 1.7	51.28 ± 1.7	60.42 ± 4.2*	39.55 ± 4.21†
Dexamethasone (10^{-7} mol/L) 72 h	27.18 ± 6.5‡	72.78 ± 6.4‡	31.62 ± 3.4‡	68.40 ± 3.4‡	54.40 ± 7.0	41.87 ± 6.7

Data are expressed as mean ± SEM.
*$P < .05$ versus live healthy.
†$P < .05$ apoptotic healthy.
‡$P < .05$ versus untreated.