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Involvement of fibrocytes in allergen-induced T cell responses and rhinovirus infections in asthma

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ABSTRACT

Allergen exposure and rhinovirus infections that propagate from the upper to the lower airways are the most frequent causes of asthma exacerbation. In patients at increased risk of disease exacerbations, chronic airway inflammation is associated with the airway recruitment of circulating fibrocytes, bone marrow-derived $CD34^+CD45RO^+CD11b^+CD13^+HLA\text{-}DR^+$ progenitors that have antigen-presenting function and fibroblast-like properties. This study demonstrates that allergen-pulsed circulating fibrocytes from patients with allergic asthma are potent inducer of the predominant release of the T helper type (Th)2 cytokines IL-4 and IL-5 from autologous naïve and memory $CD4^+$ T cells. This study also provides evidence that circulating fibrocytes from allergic asthmatics are susceptible to rhinovirus infection. Infected cells release high amounts of pro-inflammatory cytokines with minimal production of IFN-α/β. Moreover, allergen-pulsed fibrocytes support prolonged rhinovirus replication and release larger quantities of pro-inflammatory cytokines upon rhinovirus infection than unpulsed fibrocytes. Thus, fibrocytes may amplify allergen-induced, Th2 cell-driven inflammatory responses and promote further inflammation by functioning as a reservoir for rhinovirus replication in asthmatic airways. Through these mechanisms, fibrocytes may play an important role in the provocation of disease exacerbations.

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1. Introduction

Asthma is a common disorder of the airways characterized by T helper type (Th)2 lymphocyte-driven chronic inflammation [1]. Disease exacerbations are frequently induced by allergen exposure and rhinovirus (RV) infections involving both the upper and the lower airways, and there is a synergistic interaction between viral infections and allergen exposure in provoking exacerbations in allergic asthmatic subjects [1–4]. The risk of asthma exacerbations increases in patients with severe and difficult-to-treat disease [5]. In these subjects there is a marked increase in the number of circulating fibrocytes (FBs) [6–8], which are recruited to the inflamed bronchial mucosa as a consequence of the local release of FB chemoattractants and growth factors [7,8]. Elevated numbers of FBs are also present in the bronchial mucosa of allergic asthmatic patients not treated with anti-inflammatory drugs [9], and the airway recruitment of FBs increases during episodes of disease worsening provoked by natural allergen exposure [10] and after an allergen inhalation test [11]. FB accumulation has detrimental effects because these cells produce potent pro-inflammatory cytokines and may directly promote a progressive, treatment-resistant fibrotic remodeling of the bronchial wall by serving as a renewable source of collagenous and non-collagenous extracellular matrix molecules [7,10,11].

The FBs that populate the asthmatic airways most frequently localize beneath the epithelium [9,11], which is dysfunctional and more susceptible to shedding than normal epithelium and is unable to mount an effective anti-viral response to RV infection [12–14]. Therefore, subepithelial FBs are likely exposed to allergens and RVs that penetrate through the defective epithelial barrier. It has been previously reported that human and porcine fibrocytes isolated from long-term cultures of normal peripheral blood mononuclear cells (PBMCs) may function as accessory cells in the presentation of soluble and viral antigens to T lymphocytes [15,16]. The clinical relevance of these observations remains to be determined particularly in conditions where there are inherent alterations in the innate and adaptive immune responses to

Abbreviations: Ab, antibody; α-SMA, α-smooth muscle actin; COL1, type I pro-collagen; COL1A1, α1 chain of type I pro-collagen; FB, fibrocyte; HDM, house dust mite; m, monoclonal; mDC, myeloid dendritic cell; MDM, monocyte-derived macrophage; MOI, multiplicity of infections; PBMC, peripheral blood mononuclear cell; RV, rhinovirus; $TCID_{50}$, 50% tissue culture infective dose; Th, T helper type; TT, tetanus toxoid; UV, ultraviolet light.

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allergens and viral infections, such as asthma [1,2]. The present study was addressed to investigate if FBs directly isolated from the peripheral blood of individuals with persistent asthma are involved in the induction of a predominant Th2 response to clinically relevant allergens, which is a characteristic of this disease, and if they are susceptible to RV infection. Normal FBs were not tested because the number of circulating FBs is extremely low in normal individuals [6,7,10,17] and these cells do not populate normal peripheral organs, also including normal lungs [7,9]. Control myeloid cells with known antigen-presenting function and response to RV infection in humans were autologous circulating myeloid dendritic cells (mDCs) [18,19] and monocyte-derived macrophages (MDM) [20], respectively.

2. Materials and methods

2.1. Patients

FB donors were 26 non-smoking adult individuals who suffered from moderate or severe persistent asthma according to the guidelines of the Global Initiative for Asthma [21] (Supplementary information). All patients had positive skin prick tests for the house dust mite (HDM), the allergen most frequently involved in allergic asthma in Europe [22]. All of them reported a history of asthma worsening upon increased exposure to this allergen and following upper airway infections. They provided informed consent to study participation following protocol approval by the appropriate institutional review board.

2.2. Isolation and phenotypic analysis of circulating cells

The isolation of peripheral blood FBs and mDCs was performed as described elsewhere [17,23,24]. Untouched circulating $CD45^{+}$-$CD14^{+}CD16^{--}$monocytes, naïve $CD4^{+}CD45RA^{+}$ T lymphocytes and effector memory $CD4^{+}CD45RO^{+}$ T cells were isolated from PBMCs by using commercially available cell isolation kits (Supplementary information). In some experiments, $CD34^{+}$ cells were removed from the isolated $CD14^{+}CD16^{-}$ monocytes by immunomagnetic depletion with Dynabeads CD34 (Invitrogen). Phenotypic analyses by flow cytometry were performed according to previously reported procedures [10,17], as described in the Supplementary information. The expression of type I pro-collagen (COL1) at the mRNA level was evaluated in cytospin preparations by in situ hybridization, using a validated digoxigenin-labeled antisense probe (C112) [25] for the detection of the mRNA encoding the detection of the mRNA encoding α1 chain of COL1 (COL1A1) and a previously described methodology [10,11]. All the media, buffers and solutions used in this study were either endotoxin-free according to the manufacturer's certification or contained concentrations of endotoxin < 0.1 EU/ml (LAL test, Thermo Scientific).

2.3. Effects of HDM-stimulated cells on naïve and memory $CD4^{+}$ T cells

Freshly isolated $CD45^{+}CD45RO^{+}CD11b^{+}CD13^{+}CD34^{+}COL1^{+}$ FBs and $CD45^{+}CD45RO^{+}$ $CD11c^{+}CD13^{+}CD34^{-}COL1^{-}$ mDCs were incubated for 48 h in complete medium (RPMI-1640 medium supplemented with 10% human AB serum, 10 mM HEPES, 1% non-essential amino acids, 1% sodium piruvate, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin) as previously described for mDCs [23,24]. Following treatment with 25 μg/ml mitomycin C (Sigma–Aldrich) for 30 min to prevent proliferation, cells were washed repeatedly in complete medium and incubated (37 °C, 5% CO_2 humidified atmosphere) for 24 h in medium alone or in medium supplemented with either 10 μg/ml HDM (ALK-Abelló) or 5 μg/ml tetanus toxoid (TT) (Merck Millipore) as control antigen. After repeated cell washing, titrated numbers of HDM-pulsed or unpulsed FBs and mDCs were co-cultured (37 °C, 5% CO_2 humidified atmosphere) in 96-well plates for 5 days with either autologous $CD4^{+}CD45RA^{+}$ T lymphocytes or autologous $CD4^{+}CD45RO^{+}$ T cells (1 x 10^5 cells/well) in complete medium. To detect T lymphocyte proliferation, the cells were pulsed with 1 μCi/well of [methyl-^{3}H]-thymidine (PerkinElmer) during the last 12 h of the incubation period and the incorporation of [methyl-^{3}H]-thymidine into the DNA was quantified by liquid scintillation counting. The concentrations of the Th1 cytokine IFN-γ, Th2 cytokines IL-4 and IL-5, and Th17 cytokine IL-17A in the supernatants were measured with the use of ELISA kits from Abcam.

2.4. Western blot analysis of co-cultured cells

Western blot analysis of the expression of α-smooth muscle actin (α-SMA) in cell lysates was performed as previously described [10].

2.5. Response to rhinovirus infection

A member of the major group of human RVs, serotype 16, was grown in HeLa cells and prepared as described elsewhere [26,27]. Virus stocks or supernatants of infected cells were titrated on HeLa cells to determine their 50% tissue culture infective dose ($TCID_{50}$) per ml [20,26,27]. RV16 was exposed to ultraviolet light (UV) to obtain a replication-deficient, inactive control (UV-RV16) [20,26,27]. Control autologous MDMs were generated as previously reported [20]. FBs and MDMs were suspended in low-serum medium (RPMI-1640 medium supplemented with 2% human AB serum, 10 mM HEPES, 1% non-essential amino acids, 1% sodium piruvate, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin) and cultured overnight in 96-well plates. Cells were then exposed to RV16 at a multiplicity of infection (MOI) of 1 with continuous shacking [20]. After 1 h, cells were washed repeatedly to remove unattached virus and fresh low-serum medium was added to each well. Control cells were exposed to UV-RV16 or supernatants from uninfected HeLa cells (mock). All cells were then incubated (37 °C, 5% CO_2 humidified atmosphere) for up to 72 h in low-serum medium and supernatants and cell lysates were collected at various points in time. Apoptosis and necrosis were evaluated by flow cytometry analysis of cells stained with annexin-V and propidium iodide [27]. Viral replication was assessed by quantification of the viral RNA in cell lysates by real-time quantitative polymerase chain reaction procedure and signals were normalized to 18S rRNA [27]. Primer sequences were the same as previously published [27,28]. Cell supernatants were assayed for contents of IFN-α, IFN-β, IL-6, CXCL8/IL-8, and TNF-α by employing commercially available ELISA kits (R&D Systems). In some experiments, FBs were pulsed for 24 h with HDM as indicated above before being infected with RV16.

2.6. Statistical analysis

Data were analyzed by using GraphPad Prism version 5, with $\alpha = 0.05$ (Supplementary information).

3. Results

3.1. Circulating fibrocytes are distinct from mDCs and monocytes

FBs expressed HLA-DR, the co-stimulatory molecules CD80 and CD86, CD40 and ICAM-1 but differed from mDCs as they were $CD34^{+}CD11b^{+}CD11c^{-}CD14^{+}$ cells and lacked the mDC specific marker CD83 (Fig. 1A). Neither mDCs nor FBs expressed CD16 or

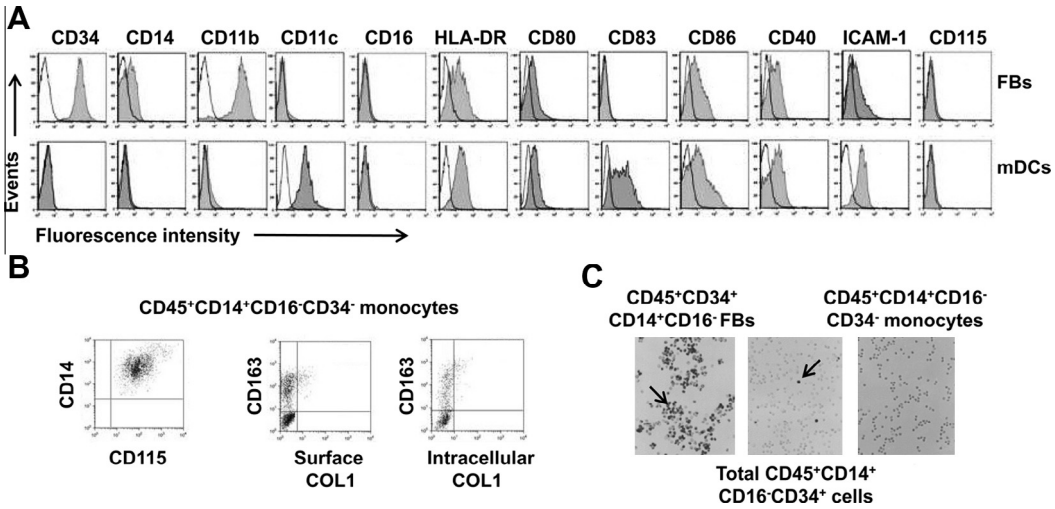

Fig. 1. Comparison of the phenotype of circulating fibrocytes (FBs), myeloid dendritic cells (mDCs) and monocytes by flow cytometry analysis of the surface and intracellular expression of the indicated markers (panels A and B) and by in situ hybridization analysis of the expression of the mRNA encoding type I pro-collagen (COL1), α1 chain (COL1A1), on cytospin preparations (panel C). (A) Each histogram shows staining with specific antibodies for the indicated markers (shaded area) and background staining with control antibodies (solid line). (B) The horizontal and vertical lines mark fluorescence intensity greater than that observed with control Abs. (C) Arrows point to cells stained with the probe for COL1A1 mRNA. Results are representative of 4–5 experiments performed with cells from different donors.

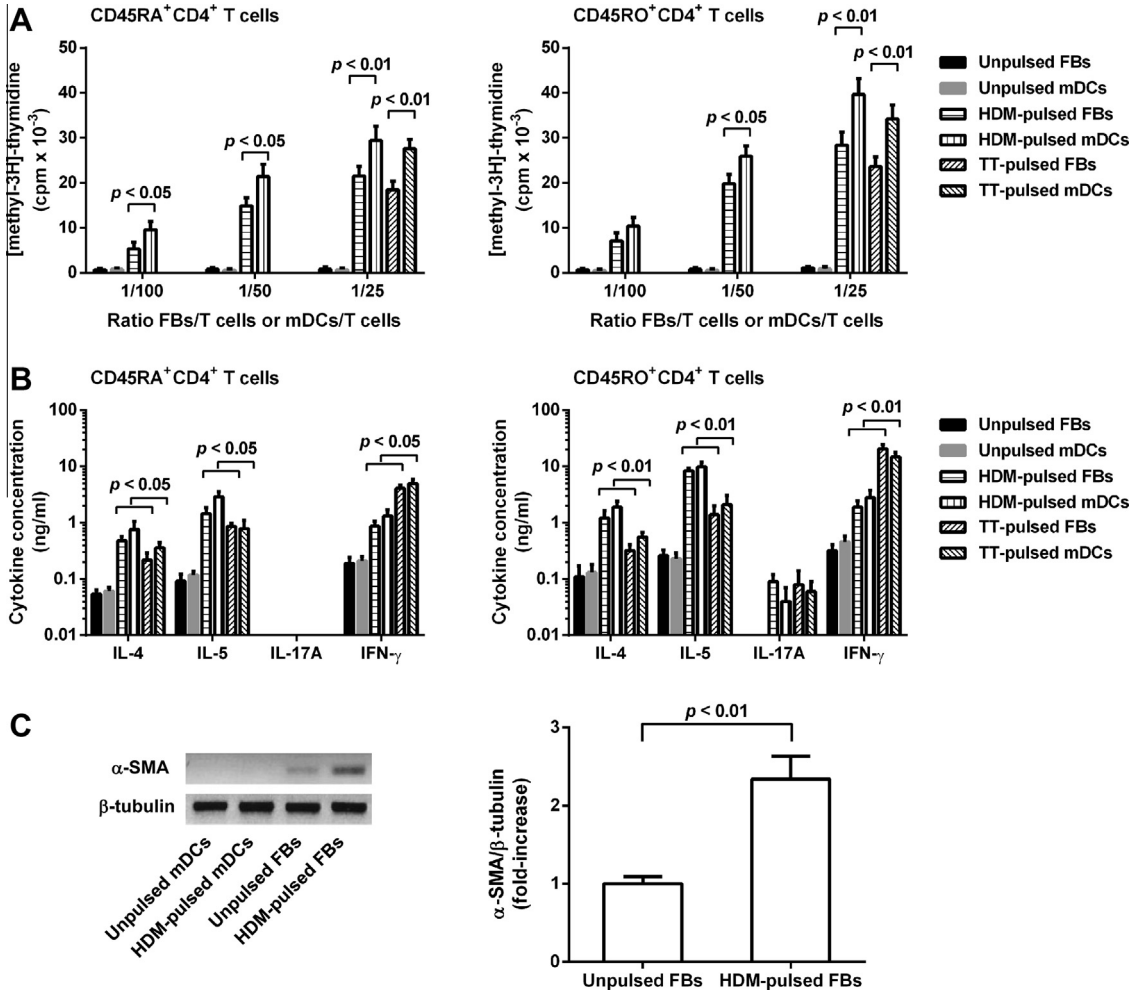

Fig. 2. Activation of naïve CD45RA$^+$CD4$^+$ T lymphocytes and memory CD45RO$^+$CD4$^+$ T cells by house dust mite (HDM)-pulsed fibrocytes (FBs) and HDM-pulsed myeloid dendritic cells (mDCs) and increased expression of α-SMA in HDM-pulsed FBs co-cultured with CD45RO$^+$CD4$^+$ T cells. (A) T cell proliferation. (B) Cytokine release by T cells. Tetanus toxoid (TT) was the control antigen. (C) Western blot analysis of the expression of α-SMA in cell lysates with a representative blot and quantitative analysis using the loading control, β-tubulin, for signal normalization. Unless otherwise indicated, the ratio FBs/T cells or mDCs/T cells was 1/25. Data are expressed as the means and SEMs ($n = 5$).

CD115, the receptor for macrophage colony-stimulating factor (Fig. 1A). CD115 was instead detected at high level on the surface of $CD45^+CD14^+CD16^-$ monocytes, which had been depleted of $CD45^+CD34^+CD14^+CD16^-$ FBs by immunomagnetic removal of all $CD34^+$ cells (Fig. 1B). A significant proportion of these cells still stained for COL1 (Fig. 1B). COL1 immunoreactivity was limited to $CD14^{bright}CD16^-$ $CD34^-$ monocytes that also stained for the transmembrane and intracellular stored scavenger receptor CD163, and was detected on the cell surface and inside the cells (after permeabilization) (Fig. 1B). CD163 is present on the cells surface of about 10% of normal circulating monocytes (mostly $CD14^{bright}CD16^-$ monocytes) but its expression markedly increases in the circulating monocytes and tissue macrophages of patients with certain fibrotic diseases, such as idiopatic pulmonary fibrosis [29]. CD163 and other scavenger receptors expressed by the same cells contain a collagenous domain identical to the triple-helix domain of most fibrillar collagens [30,31] and may stain with anti-COL1 antibodies. This possibility was addressed by in situ hybridization analysis of the expression of COL1A1 mRNA on cytospin preparations, using a validated digoxigenin-labeled antisense probe. As shown in Fig. 1C, COL1A1 mRNA was clearly detected in isolated FBs and in some of the total $CD45^+CD14^+CD16^-CD34^+$ PBMCs. Following removal of the $CD34^+$ cells ($CD34^+CD14^+CD16^-$ FBs) from this population, the residual $CD45^+$ $CD14^+CD16^-CD34^-$ monocytes were invariably negative for the expression of COL1A1 mRNA (Fig. 1C).

3.2. HDM-pulsed FBs are potent inducers of the release of the Th2 cytokines

HDM-pulsed FBs were significantly less effective than HDM-pulsed mDCs at inducing the proliferation of autologous naïve $CD45RA^+CD4^+$ T cells and memory $CD45RO^+CD4^+$ T cells (Fig. 2A). However, HDM-pulsed FBs and HDM-pulsed mDCs similarly induced a predominant release of the Th2 cytokines IL-4 and IL-5 from naïve and memory $CD4^+$ T cells when compared to TT-pulsed FBs and TT-pulsed mDCs (Fig. 2B). Detectable levels of IL-17A were only produced by memory $CD4^+$ T lymphocytes that had been co-cultured with HDM- or TT-pulsed FBs and mDCs (Fig. 2B). Because Th2 cytokines [10] and IL-17A [10,32] are known to increase the expression of α-SMA in FBs, the contents of intracellular α-SMA in HDM-pulsed and unpulsed FBs similarly co-cultured with memory $CD4^+$ T lymphocytes were compared by western blot analysis of cell lysates. Allergen-specific activation of memory $CD4^+$ T lymphocytes was associated with an enhanced expression of the contractile protein in co-cultured HDM-pulsed FBs (Fig. 2C). No expression of α-SMA was detected in the control co-cultures of HDM-pulsed mDCs and memory $CD4^+$ T cells (Fig. 2C).

3.3. FBs are susceptible to RV16 infection and infected cells release high amounts of pro-inflammatory cytokines

RV16 entered and replicated in FBs, as demonstrated by the exponential increase in the production of viral RNA in these cells. Viral RNA levels were still significantly increased at 48 h but not at 72 h post infection (Fig. 3A). RV16 titers in the supernatants peaked at 24 h post infection and slowly declined thereafter (Fig. 3B). In keeping with previous observations [20], RV16 did not replicate efficiently in control MDMs (Figs. 3A and B). FBs and MDMs also differed in the pattern of cytokine release upon RV16 infection. FBs produced larger amounts of the pro-inflammatory cytokines IL-6, CXCL8/IL-8, and TNF-α than MDMs (Fig. 3C). Moreover, relatively high levels of the type I IFNs with anti-viral properties, IFN-α and IFN-β, were present in MDM supernatants while very low amounts of these cytokines were detected in FB supernatants (Fig. 3C). In both cell types, induction of the release of pro-inflammatory cytokines was largely dependent on virus replication because infection with UV-RV16 resulted in a minimal increase in the production of IL-6, CXCL8/IL-8, and TNF-α over the constitutive release observed in mock-treated cells (Fig. 3C).

3.4. HDM-pulsed FBs are more susceptible to RV16 infection and show enhanced release of pro-inflammatory cytokines

FBs cultured for 24 h in the presence of HDM showed increased expression of ICAM-1, the RV16 receptor [33], in comparison with FBs cultured for 24 h in medium alone (Fig. 4A). After RV16 infection, viral replication (Fig. 4B) and virus release (Fig. 4C) were more intense and lasted longer in HDM-pulsed FBs than in unpulsed FBs. RV16 infection provoked cell death to a greater extent in cultures of HDM-pulsed FBs than in cultures of unpulsed FBs (Fig. 4D). The RV16-induced release of IL-6, CXCL8/IL-8, and TNF-α was more marked in HDM-pulsed FBs than in unpulsed cells (Fig. 4E). The supernatants of HDM-pulsed and infected FBs contained only trace amounts of IFN-α and IFN-β (Fig. 4E).

4. Discussion

This study demonstrates that circulating FBs from patients with moderate and severe allergic asthma are distinct from mDCs and

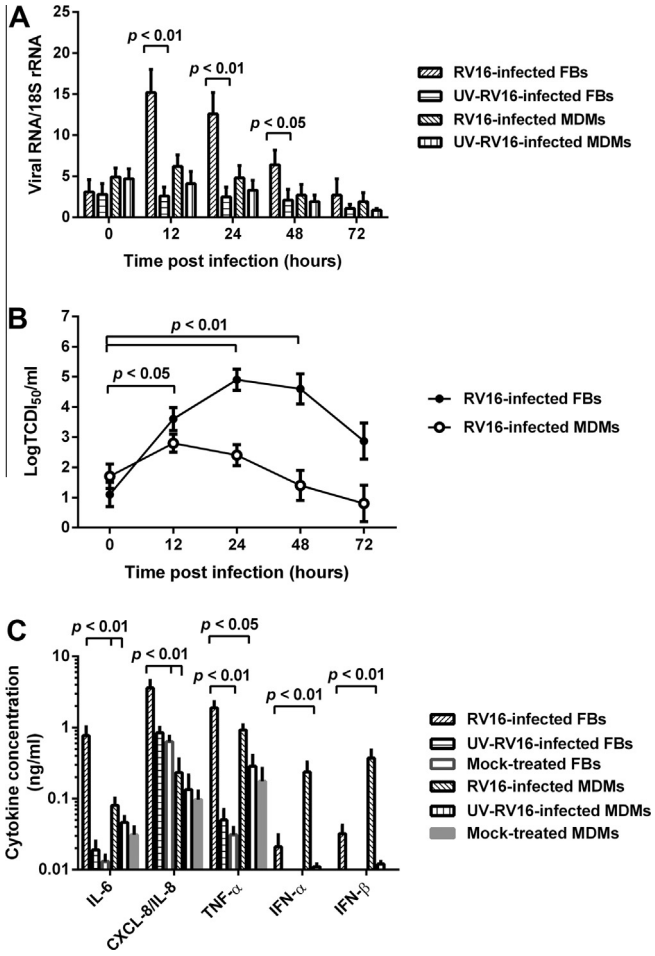

Fig. 3. Response of fibrocytes (FBs) and monocyte-derived macrophages (MDMs) to infection with rhinovirus serotype 16 (RV16). (A) Viral RNA levels at the indicated points in time post infection. (B) RV16 titers measured in the supernatants at the same points in time post infection. (C) Release of pro-inflammatory cytokines and anti-viral type I IFNs over 72 h post infection. Mock-treated cells and/or cells infected with ultraviolet-inactivated RV16 (UV-RV16) were used as controls. Data are expressed as the means and SEMs (n = 4–5).

monocytes and may be specifically involved in the immune responses to the clinically relevant allergen, HDM. Although HDM-pulsed FBs are less potent than HDM-pulsed mDCs at inducing the proliferation of autologous naïve and memory CD4$^+$ T lymphocytes, they similarly induce the predominant release of the T helper type (Th)2 cytokines IL-4 and IL-5 from activated CD4$^+$ T cells. In keeping with previous observations [34], TT-pulsed mDCs from allergic asthmatics induce the predominant release of the Th1 type cytokine IFN-γ from T cells, and TT-pulsed FBs have similar effects. Because there is an increased accumulation of FBs in the airways of patients with allergen-induced asthma exacerbation [10,11], these cells may work in concert with resident DCs and migratory mDCs to amplify allergen-induced, Th2 cell-driven inflammatory responses in the bronchial mucosa at every allergen exposure. Moreover, high numbers of fibrocytes are present in the airways of patients with chronically severe and difficult-to-treat asthma [7,8]. In these patients, bronchial FBs may favor the persistence of the inflammatory infiltrate, if the individuals are continuously exposed to the allergens to which they are sensitized, or contribute to precipitate an acute exacerbation by increasing inflammation after a transient increase in allergen exposure. The enhanced expression of α-SMA in FBs pulsed with HDM and co-cultured with allergen-specific memory CD4$^+$ T cells is an interesting finding because clusters of proliferating T cells and immature α-SMA cells, with a FB-like morphology, have been detected in the airways of atopic patients with moderate or severe asthma [35].

This study also provides evidence that circulating FBs from allergic asthmatics are susceptible to infection with a member of the major group of the RVs implicated in acute exacerbation of asthma [2–4]. Infected cells support sustained viral replication and release large amounts of IL-6, CXCL8/IL-8, and TNF-αwith minimal production of the type I IFNs IFN-α and IFN-β. Studies reviewed elsewhere [10] suggest that FB-derived IL-6, CXCL8/IL-8, and TNF-α may worsen airway inflammation in asthma and that IL-6 may further promote the imbalance between Th2 cells and regulatory T cells in the bronchial mucosa of allergic asthmatic individuals. The release of type I IFNs from RV-infected cells is critical for the development of an adequate anti-viral immune response because these cytokines induce the recruitment of NK and cytotoxic T cells to the infected tissue site and restrict virus replication in the infected cells [36]. IFN-mediated responses are deficient in asthma [14] and the impaired production of type I IFNs

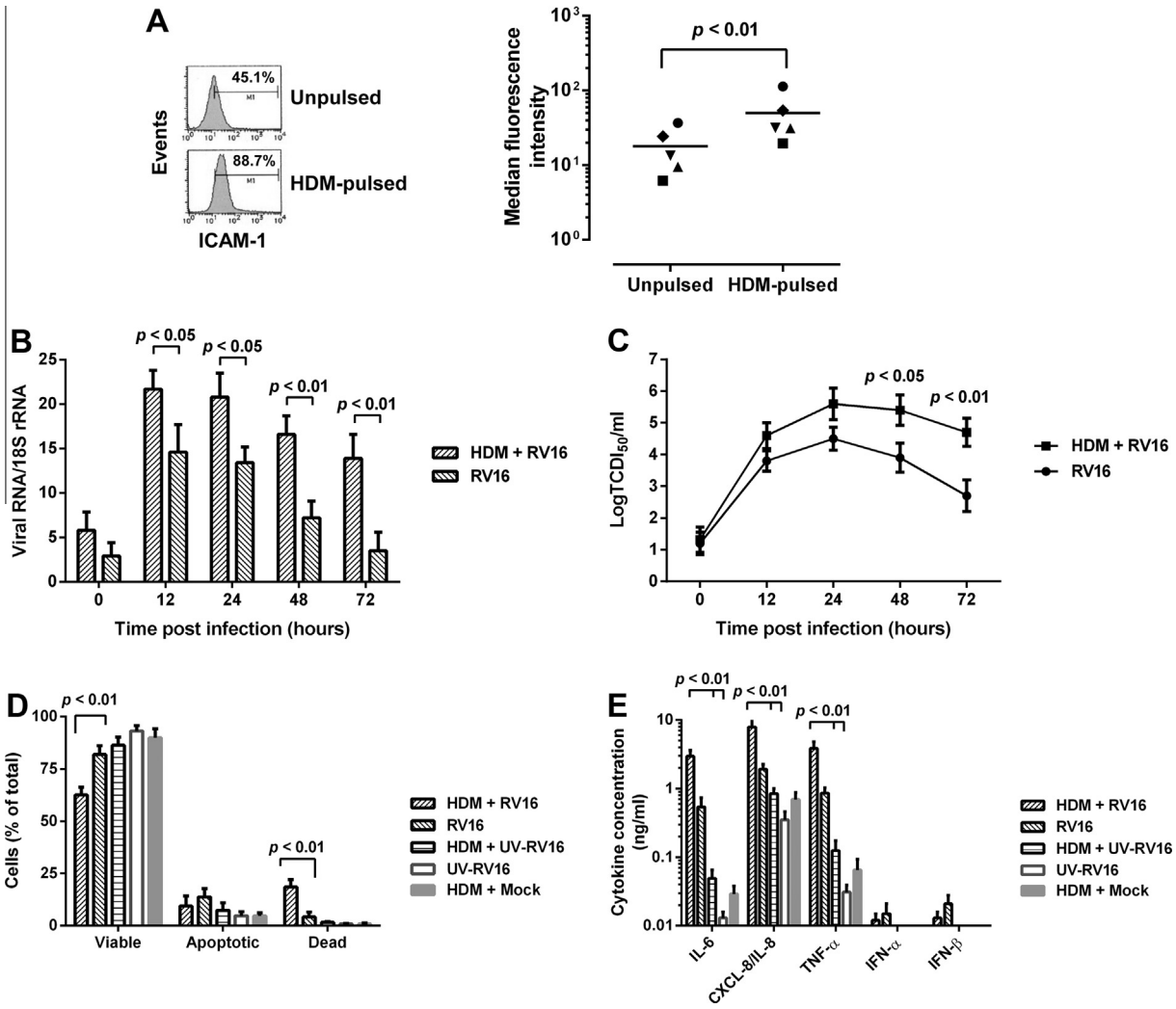

Fig. 4. Comparison of the responses to infection with rhinovirus serotype 16 (RV16) of fibrocytes (FBs) pulsed with house dust mite (HDM) and unpulsed FBs. (A) Representative histograms showing flow cytometry analysis of the expression of the receptor for RV16, ICAM-1, on the surface of HDM-pulsed and unpulsed FBs. The numbers inside the histograms are the percentages of cells specifically stained with the monoclonal antibody against ICAM-1. The graph shows a comparison of the median fluorescence intensities from 5 experiments with the mean values (horizontal line). (B) Viral RNA levels at the indicated points in time post infection. (C) RV16 titers measured in the supernatants at the same points in time post infection. (D) Analysis of the frequency of viable, apoptotic and dead cells at 72 h post infection. (E) Release of pro-inflammatory cytokines and anti-viral type I IFNs over 72 h post infection. Control unpulsed and HDM-pulsed cells were either mock treated or infected with ultraviolet-inactivated RV16 (UV-RV16). In panels B–E, the data are expressed as the means and SEMs (n = 4–5).

from infected FBs may contribute to explain the persistence of RVs in asthmatic airways.

In the present study, HDM-pulsed FBs were more susceptible to RV infection than unpulsed FBs. The enhanced expression of the RV16 receptor in HDM-pulsed FBs may have facilitated virus entry, as demonstrated by the increased percentage of dead cells in cultures of HDM-pulsed and infected FBs. Because the magnitude of the release of pro-inflammatory cells was largely dependent on the intensity of virus replication, HDM-pulsed FBs released larger amounts of pro-inflammatory cytokines upon RV infection than unpulsed FBs. The fact that HDM-pulsed and infected FBs produced levels of type I IFNs even lower that unpulsed and infected cells may explain at least in part the prolonged replication of RV16 in these cells [36].

In conclusion, the results of this study strongly suggest that FBs may amplify allergen-induced, Th2 cell-driven inflammatory responses and promote further inflammation by functioning as a reservoir for RV replication in asthmatic airways. Through these mechanisms, FBs may therefore play an important role in the provocation of disease exacerbations in allergic asthma. The findings of the present study may also be relevant to other conditions characterized by Th2-driven inflammation, increased susceptibility to infections and increased recruitment of FBs to the affected skin, such as chronic atopic dermatitis [1,37].

Disclosures

S. Mattoli is founding shareholder and director of AVAIL GmbH. The co-authors have no potential conflicts of interest or financial interests.

Author contributions

All authors substantially contributed to the conception and design of this study, or collection of data, or analysis and interpretation of data; drafting or critically revising the manuscript; approval of the paper submitted for publication.

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FibroGENE and CellNet projects (no involvement in study conduction and manuscript preparation).

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.2013.06.099.

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Supplementary information

Materials and methods

Patients

Fibrocyte (FB) donors were 26 non-smoking adult patients (14 females; mean age ± SEM: 43.8 ± 3.9 years) with moderate or severe persistent asthma and a history of disease worsening following infections of the upper airways and upon increased exposure to the allergen to which they were sensitized, the house dust mite (HDM). Asthma was defined on the basis of the guidelines of the Global Initiative for Asthma (GINA) [1] and disease severity was classified on the basis of the treatment required to reduce as much as possible the frequency and intensity of symptoms (GINA treatment steps) [1]. On functional evaluation by spirometry, the mean (± SEM) forced expiratory volume in 1 second was 80.1% (± 4.3%) of predicted and increased by 9.6% (± 3.8 %) following the inhalation of a short-acting bronchodilator (400 μg of salbutamol). The mean forced expiratory volume in 1 second/forced vital capacity ratio was 71.6% (± 3.1%). The results of a standard skin prick tests confirmed that all patients were sensitized to the HDM *Dermatophagoides pteronyssinus*. Patients had not suffered from respiratory or systemic infections in the previous 2 months and had not been treated with systemic corticosteroids or other anti-inflammatory drugs for at least 3 months. All of them were receiving inhaled corticosteroids in combination with long-acting bronchodilators two times daily and could use rapid-acting bronchodilators on an as-needed basis as symptom relievers (GINA treatment steps 3 and 4) [1]. For the isolation of circulating cells, patients were asked to avoid reliever use for 24 hours and venous blood samples were

collected before the morning dose of inhaled medications, at a distance of 3-4 days from skin prick testing and spirometry.

Isolation and phenotypic analysis of circulating cells by flow cytometry

The isolation of peripheral blood $CD34^+CD45RO^+CD11b^+CD13^+$ FBs was performed as described elsewhere [2] and the purity (≥ 93%) of the isolated population was demonstrated by flow cytometry analysis of cells stained for intracellular type I pro-collagen (COL1) [2]. Myeloid dendritic cells (mDCs) were initially isolated from peripheral blood mononuclear cells (PBMCs) by negative immunomagnetic selection of the lineage (CD3, CD14, CD16, CD19, CD20, CD56) negative, CD1c/BDCA-1^+ and CD141/BDCA-3^+ fractions [3], [4], using the Myeloid Dendritic Cell Isolation Kit from Miltenyi Biotec. The recovered cells were stained with PerCP-conjugated monoclonal (m) antibody (Ab) against HLA-DR and PE-conjugated mAb against CD11c (both from BD Biosciences) and sorted with a BD FACSAria II (BD Biosciences) to obtain a 93-98% pure population of total mDCs [3, 4]. The isolation of untouched $CD14^+CD16^-$ monocytes (purity ≥ 98%) from total $CD45^+$ PBMCs was performed by using the Monocyte Isolation Kit II from Miltenyi Biotec to deplete T cells, B cells, NK cells, and DCs, followed by immunomagnetic removal of $CD34^+$ FBs with Dynabeads CD34 (Invitrogen). Untouched naïve $CD4^+CD45RA^+$ T lymphocytes and effector memory $CD4^+CD45RO^+$ T cells (purity > 97%) were isolated from PBMCs by using the specific cell isolation kits from Miltenyi Biotec. Phenotypic analyses by flow cytometry were performed as previously described [2, 5]. Cells were initially treated with FcR blocking reagent (Miltenyi Biotec) and then incubated with the following Abs against specific

markers or the appropriate controls (isotype matched IgGs for mAbs or irrelevant Abs): FITC- or PE-conjugated anti-CD163 mAb (GH1-61, Santa Cruz Biotechnology), anti-CD11b mAb (Abcam), anti-CD14 and anti-CD45 (BD Biosciences) mAbs; FITC-conjugated anti-CD11c (DAKO), anti-HLA-DR (Diatec Monoclonals), anti-CD14, anti-CD18, anti-CD80, anti-CD83 and anti-CD86 (Beckman-Coulter) mAbs, anti-CD16 mAb (BD Biosciences) and mAb against the adhesion molecule and major rhinovirus receptor [6], ICAM-1/CD54 (BD Biosciences); APC-conjugated anti-CD34 (StemCell Technologies) mAb; unconjugated anti-CD115/macrophage colony-stimulating factor receptor mAb and FITC-conjugated secondary Ab (Santa Cruz Biotechnology). For detection of intracellular type I pro-collagen (COL1), cells were fixed and permeabilized with Cytofix/Cytoperm kit (BD Biosciences) before being sequentially incubated with rabbit anti-COL1 Ab (Rockland) and a FITC-labeled secondary Ab (Invitrogen) or directly treated with a mouse anti-COL1 (LifeSpan BioSciences) mAb that had been conjugated to FITC using the EasyLink FITC Conjugation Kit (Abcam).

Statistical analysis

Data were analyzed by the paired or unpaired Student's t test, one-way ANOVA followed by Dunnett's or the Tuckey's post-hoc test, or two-way ANOVA followed by the Bonferroni post-hoc test for multiple comparisons, as appropriate, using the software GraphPad Prism version 5 for Windows and the associated GraphPad Prism Statistics Guide (GraphPad Software Inc., La Yolla, CA, USA). In all cases, the probability of incorrectly rejecting the null hypothesis when it was actually true was $\alpha = 0.05$.

Therefore, a *p*-value of less than 0.05 was considered to be statistically significant. Results are reported as the means and SEMs.

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